THE UNIVERSITY *of* EDINBURGH

A BETTER UNDERSTANDING OF CANINE TELOMERASE AND ITS POTENTIAL APPLICATIONS IN CANINE ONCOLOGY

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DECLARATION

In accordance with the regulations of the university, I declare that this thesis has been completed entirely by me, and that the work presented is my own, except where acknowledgement has been made in the text.

This work has not been submitted for any other degree or professional qualification.

Signed:

Date:

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ABSTRACT

Telomerase, discovered in 1985, is considered a near-universal marker of malignancy and therefore has a potential use in cancer therapeutics and diagnostics. In this study, I used several approaches to gain a better understanding of telomerase and its potential applications in the canine context, for both cancer therapeutics and diagnosis. Having already developed an effective siRNA viral vector *in vitro*, the challenge still remained to deliver it efficiently *in vivo*. Thus, I initially investigated two possible approaches for *in vivo* delivery. First, I investigated a cell-based system for direct delivery to the tumours. Specifically I optimised a system for efficient gene-transfer to endothelial cells using a green fluorescent protein plasmid vector, and monitored systemic delivery by *ex vivo* imaging of dye-labelled cells in a canine xenograft tumour mouse model. In parallel, *in vitro* I investigated the gene transfer mediated by a novel dendrimer vector that can form nanoparticles with DNA and accumulate in tumour sites *in vivo* after i.v. administration. In order to utilize these delivery systems, I developed a DNA plasmid-based siRNA vector and tested its efficacy on canine tumour cells.

To investigate telomerase as a cancer biomarker, I conducted a study that aimed to detect circulating telomerase reverse transcriptase (TERT) mRNA in serum taken from canine cancer patients. For this I developed several systems for effective RNA isolation from serum and used both conventional and quantitative PCR assays to detect TERT expression. Although for the first time I can confirm the existence of mRNA in serum of canine cancer patients, in this clinical study, I could only detect telomerase transcripts in a very small proportion of canine cancer patients.

In a final pilot study to investigate anti-ageing technologies, I looked at the potential for drug-dependant telomerase induction rather than inhibition. For this I investigated the ability of three candidate drugs to induce TERT mRNA activation in canine embryonic fibroblasts. In this study, telomerase induction was measured using the quantitative PCR method that I had developed for serum detection.

In summary, I have demonstrated that a cell-based delivery vehicle has a potential application in cancer therapy, but that more development is required before it can be applied clinically. I have also reported here that PPIG3 dendrimer-based gene transfer *in vitro* is low in canine cancer cells and thus require more optimisation and development before it can be utilised as an efficient systemic delivery vehicle. For the siRNA experiment, unfortunately, I did not observe any telomerase gene-silencing in canine cancer cells using the plasmid-based siRNA expression vector, and therefore the gene sequence of cTR that we were targeting as well as the siRNA plasmid-vector that we used needs further validation in canine cells. I also suggest that TERT mRNA may not be a good serum biomarker for canine cancer diagnostics as I did not find TERT transcript in most of our serum samples from canine cancer patients, although circulating mRNA of a housekeeping gene was detected. Finally, in a pilot study, I have demonstrated that telomerase can be induced in normal canine somatic cells using small molecules. However, the long-term effects of telomerase induction on ageing must be determined in future studies.

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ABBREVIATIONS

293FT	Transformed Human Embryonic Kidney Cells
5T4	or TPBG, Trophoblast Glycoprotein
AFP	α-fetoprotein
AP-1	Activator Protein-1
ATM	Ataxia-telangiectasia Mutated
ATR	Ataxia Telangiectasia- and Rad3-related
bp	Base Pair
CdCS	Cri Du Chat Syndrome
cDNA	Complementary DNA
CEA	Carcinoembryonic Antigen
CEF	Canine Embryonic Fibroblasts
CHK1	Checkpoint Kinase 1
CHK2	Checkpoint Kinase 2
C-myc	v-myc myelocytomatosis viral oncogene homologue (avian)
CXCR4	C-X-C Chemokine Receptor Type 4
D-17	Canine Osteosarcoma Cell line
D-Loop	Displacement Loop
D-MEM	Dulbecco's Modified Eagle Medium
DKC1	Dyskerin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
Dnase	Deoxyribonuclease
DTT	Dithiothreitol
dsRNA	Double-stranded RNA
E. Coli	*Escherichia coli*
EPC	Endothelial Progenitor Cells
EPR (effect)	Enhanced Permeability and Retention (effect)
ERK	Extracellular Signal-regulated Kinase
EOMA	Murine Haemangioendothelioma Cells
Ets	E-twenty-six Protein
FBS	Foetal Bovine Serum
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GFP	Green Fluorescent Protein
G-quadruplex	Guanine-quadruplex
Gy	Gray
H1299	Human Lung Cancer Cells
HER2/neu	Human Epidermal growth factor Receptor 2

hnRNP B1	Heterogeneous Nuclear Ribonucleoprotein B1
HSV	Herpes Simplex Virus
HUVEC	Human Umbilical Vein Endothelial Cells
i.v.	Intravenously
IC50	Half Maximal Inhibitory Concentration
IPS (cells)	Induced Pluripotent Stem (cells)
kb	Kilobase
LB	Lysogeny Broth
MAD1	MAX dimerization protein 1
MEK	MAPK/ERK
MDM2	Murine Double Minute 2
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
MSC	Mesenchymal Stem Cells
NEAA	Non-essential Amino Acids
NF-water	Nuclease-Free Water
NHP2	or NOLA2, Nucleolar Family A, Member 2
NOD/SCID	Non-obese Diabetic / Severe Combined Immunodeficiency
NOP10	or NOLA3, Nucleolar Family A, Member 3
nt	Nucleotide(s)
P16	Protein 16
P21	Protein 21
P53	Protein 53
PACT	PKR-activating Protein
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
PI3K	Phosphatidylinositol 3-kinase-like Kinase
PKR	Protein Kinase R
POT1	Protein Protection of Telomeres 1
PPIG3	Polypropylenimine Generation 3
PSMA	Prostate Specific Membrane Antigen
R2D2	Double-stranded RNA-binding-domain Protein
Rap1	Repressor Activator Protein 1
Ras	Rat Sarcoma
Rb	Retinoblastoma Protein
RISC	RNA-induced Silencing Complex
RLC	RISC-loading Complex
RNA	Ribonucleic Acid
RNAi	RNA Interference
RNase	Ribonuclease
RT-PCR	Reverse Transcription Polymerase Chain Reaction

SB-HSA	Canine Haemangiosarcoma Cell Line
SB-HSA-Luc	Stably-transfected SB-HSA Cells Expressing Luciferase
SDF-1	Stromal Cell-derived Factor-1
shRNA	Short-hairpin RNA
siRNA	Small-interfering RNA
S.O.C (medium)	Super Optimal Broth with catabolite repression
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
T-Loop	Tail Loop
TAE Buffer	Tris-acetate-EDTA Buffer
TCAB1	Telomerase Cajal Body Protein 1
TERT	Telomerase Reverse Transcriptase
TIN2	TRF1-interacting Nuclear Factor 2
TPP1 or TINT1, PTOP, PIP1	Telomeric POT1-interacting Protein 1
TR or TERC	Telomerase RNA Component
TRAP	Telomere Repeat Amplification Protocol
TRBP/TARBP2P	Double-stranded RNA-binding Protein
TRF1	Telomeric Repeat Binding Factor 1
TRF2	Telomeric Repeat Binding Factor 2
TS	Non-telomeric Substrate Primer
UPRT	Uracil Phosphoribosyl Transferase
µm	micrometre

CHAPTER 1

INTRODUCTION

1.1 Cancer Burden

Despite recent advances in cancer diagnosis and treatment (Petrelli et al., 2009), generally human cancer patients still face high morbidity and mortality rates. According to Cancer Research UK Annual Report 2009, more than 293,000 people are diagnosed with cancer each year in Britain, and in 2007 there were more than 155,000 cancer patient deaths in the United Kingdom alone. Global deaths from cancer were 7.4 million in 2004 according to the World Health Organization (http://www.who.int/cancer/prevention/en/). Our canine friends are also affected: it is estimated that between one in three and one in four dogs will develop some form of cancer during their lifetime (Argyle and Khanna, 2006). These statistics show the urgent need for ongoing research in order to better understand and treat this deadly disease. Currently, cancer is considered as an accumulation of both inherited and acquired genetic mutations that cause the uncontrollable and continuous growth of abnormal cells in a living body. Visually, a tumour is a complex tissue composed of not only tumour cells but also of extracellular matrix components, fibroblasts, immune cells and endothelial cells. Generally, all cancers share six common hallmarks: cell division independent of growth signals; insensitivity to growth-inhibitory signals; inability to induce programmed cell death; limitless replicative potential; sustained angiogenesis; and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Of these distinct features, the inappropriate activation of cell signalling pathways alone is not sufficient to enable cancer cells to grow indefinitely and the limitless replicative potential does appear to be an independent mechanism that enables the indefinite growth of cancer cells, and the key cellular factor underlying this mechanism is telomerase (Hanahan and Weinberg, 2000). Telomerase was firstly discovered in protozoa, and its biological function is to synthesize and add specific repeat sequences of non-coding DNA onto chromosome ends or telomeres (Greider and Blackburn, 1985).

1.2 The Biology of Telomeres and Telomerase

1.2.1 Telomeres

Telomeres are located at the end of chromosomes and are composed of double-

stranded TTAGGG DNA repeats with a single-stranded 3' $(TTAGGG)_n$ overhang, all bound by specialized proteins. These structures maintain genome stability (O'Sullivan and Karlseder, 2010) by preventing chromosome end-to-end fusions (Tusell et al., 2008), and also by preventing the ends of chromosomes from being recognized as double-stranded DNA (dsDNA) breaks that could induce unscheduled DNA repair (Khanna and Jackson, 2001). Chromosome ends are in fact shielded by telomeric DNA and six associated proteins (Table 1), which together form the telomeric shelterin complex that is crucial for telomere stability (Martinez and Blasco, 2011). During the non-mitotic phase of the cell cycle, the shelterin complex helps the telomere to fold correctly to form a closed structure containing two internal loops, the D-loop and the T-loop (Tail loop), that stabilize and protect the telomere (Greider, 1999). The T-loop is thought to be formed by the 3'-telomeric overhang folding back and invading the duplexed telomeric repeat sequence (Griffith et al., 1999), and the D-loop (displacement loop) is thought to result from base-pairing of the 3' telomeric overhang with one internal strand after this invasion process occurs (Figure 1). In this way a structure is created that is distinct from a broken DNA end (Greider, 1999), and is not recognized as such. The telomeric sequence TTAGGG is evolutionarily conserved in all vertebrates (Meyne et al., 1989). The length of 3' overhang in human cells can vary from 24 nucleotides to over 400 nucleotides (Cimino-Reale et al., 2001).

TRF1	Telomeric Repeat Binding Factors 1
TRF2	Telomeric Repeat Binding Factors 2
POT1	Protein Protection of Telomeres 1
RAP1	Repressor Activator Protein 1
TPP1	also known as TINT1, PTOP or PIP1
TIN2	TRF1-interacting Nuclear Factor 2

Table 1 - The Shelterin Proteins of Human Telomere

The telomeric shelterin protein complex consists of TRF1, TRF2, POT1, RAP1, TPP1 and TIN2.

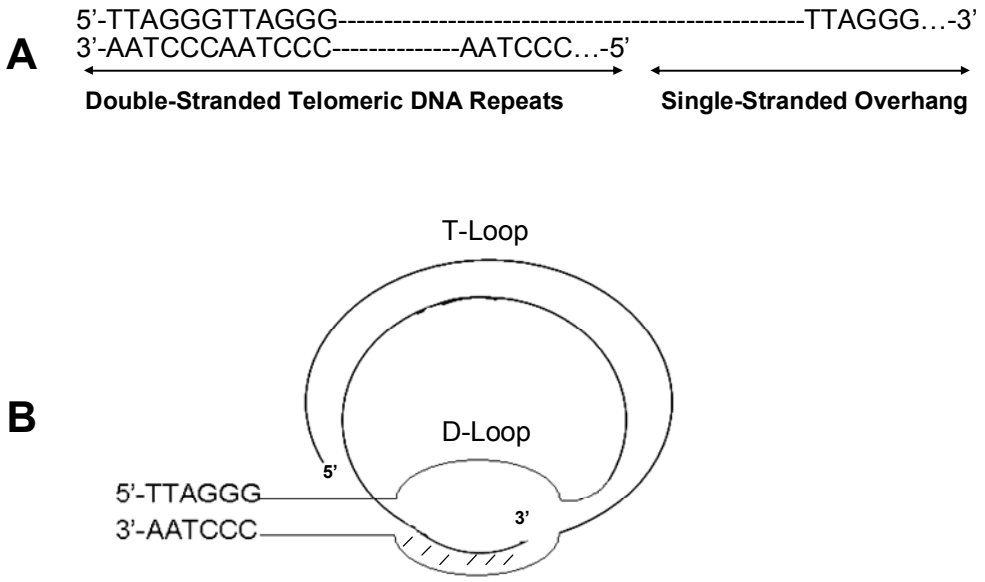


Figure 1 – The Structure of Telomere – Schematic

adapted from (Cooper, 2000)

A – Telomere which is located at the very end of chromosome, contains dsDNA repetitive TTAGGGs and ssDNA repetitive TTAGGGs overhang.

B – Telomere folds internally and creates a D-loop and a T-loop to avoid being recognized as DNA breaks and thus maintains chromosome stability.

1.2.2 Cell Senescence

The telomere not only maintains the basic structure and integrity of the chromosome, but is also responsible for regulating cell division and cellular replicative senescence, by functioning as a mitotic clock (Collado et al., 2007). The DNA end-replication problem, whereby DNA polymerase cannot copy the extreme 5' end of chromosome (Watson, 1972), results in the telomeres shortening (Olovnikov, 1996) by a rate of, for example, approximately 50bp per cell division cycle in human fibroblasts (Harley et al., 1990). It was initially observed that normal somatic cells would enter into a senescence phase after a certain number of cell divisions, and this was termed the Hayflick Limit (Hayflick and Moorhead, 1961). Cellular replicative senescence was originally characterized by a large, flattened and vacuolated cell morphology; senescence-associated β-galactosidase activity (Dimri et al., 1995); a lack of response to mitogens; and a permanent growth arrest in the G1 phase of the cell cycle

(Stein and Dulic, 1995). It was later found that replicative senescence is also associated with telomere shortening (Harley et al., 1990) and shown that cellular senescence can be triggered by shortened telomeres (Blackburn, 2001). These shortened telomeres are recognized as dsDNA breaks and are recognized by sensor proteins that activate the DNA damage response machinery (d'Adda di Fagagna et al., 2003) (Figure 2). The DNA damage pathway firstly activates phosphatidylinositol 3-kinase-like kinases (PI3Ks), such as ATM (ataxia telangiectasia mutated) or ATR (ataxia telangiectasia and Rad3-related) (Guo et al., 2007, Herbig et al., 2004). Once activated, these kinases phosphorylate downstream factors, including Checkpoint kinase 1 (CHK1) and Checkpoint kinase 2 (CHK2), which subsequently target p53 (Latif et al., 2004, Gire, 2004). The phosphorylation of p53 inhibits binding of the murine double minute 2 (MDM2) protein and thus releases p53 from ubiquitination-mediated degradation, resulting in its activation. Activated p53 then induces expression of cyclin-dependant kinases (CDKs) inhibitor p21, which inhibits cell cycle progression and initiate replicative senescence by inhibiting CDKs thus activating protein RB (pRb) by phosphorylaiton (Ben-Porath and Weinberg, 2005). When pRb is hypophosphorylated, it binds to and represses the functions of E2F family of transcriptional factors, which thus decreases the expression of many E2F target genes and results in cell cycle arrest (Maehara et al., 2005). Current knowledge indicates that p53 and pRb are required not only for the onset of cellular senescence, but also for the maintenance of the senescence program, as inactivation of these proteins during cellular senescence results in reversal of the senescent phenotype (Beausejour et al., 2003). Apart from P21, P16 is another CDKs inhibitor that can induce the onset of cellular senescence (Ruas and Peters, 1998) which becomes irreversible if a sustained period of p16 expression occurs (Dai and Enders, 2000). It is indicated that p16 may have a p53-independent senescent response towards telomere dysfunction and this mechanism may be a secondary effector of the telomere DNA damage response, after the primary p53-dependent mechanism (Jacobs and de Lange, 2004), but its involvement in telomere dysfunction-induced senescence is still being debated (Herbig et al., 2004, Smogorzewska and de Lange, 2002). In fact, both the p16-pRb and p53-p21 pathways are reported to be frequently inactivated in human cancers reflecting the importance of these proteins as tumour

suppressors. However, the mechanism for determining whether cells undergo senescence or apoptosis in response to telomere-induced DNA damage response is still not fully understood (Deng et al., 2008). In addition to causing cell cycle arrest, telomeres lacking telomeric protein TRF2 can also induce p53- and ATM-dependent apoptosis (Karlseder et al., 1999), and an increased level of apoptosis in proliferative cells was observed in a mouse model containing dysfunctional telomeres (Attardi, 2005).

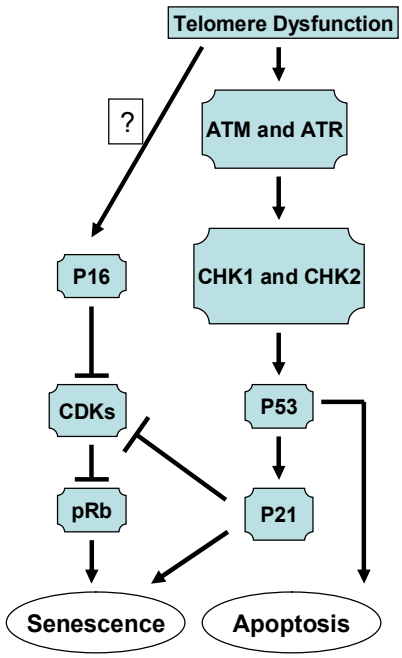


Figure 2 – Telomere Dysfunction Activates the p53 and pRb Pathways

adapted from (Deng et al., 2008)

Telomere dysfunction induces senescence via p53-p21 and p16-pRb pathways. P16-pRb pathway is less clear. Telomere dysfunction can also cause p53-dependant apoptosis.

1.2.3 Telomerase

Since its discovery telomerase has been linked with tumour immortalisation, and this enzyme has a critical role in preventing telomere loss in cancer cells (Counter et al., 1992), thereby helping them to escape from cellular replicative senescence and apoptosis (Harley, 2008). Normal somatic cells are telomerase-negative and only

limited telomerase activity is found in stem cells (Hiyama and Hiyama, 2007) and germline cells (Kim et al., 1994), thus making telomerase a tumour-specific marker. In contrast, the vast majority (approximately 85%) of human cancer cells are telomerase-positive, which identifies telomerase as a near universal tumour marker (Kim et al., 1994, Shay and Bacchetti, 1997). In support of this, one report showed that 98 out of 100 immortal cell lines, representing 18 different human tissues, and 90 out of 101 tumour biopsies, representing 12 human cancer types, were telomerase-positive, while all human somatic cells and tissues tested were telomerase-negative (Kim et al., 1994). In addition, some cancer cells (approximately 15%) can maintain telomere length in the absence of active telomerase using the DNA recombination-based mechanism, alternative lengthening of telomere (ALT) (Cesare and Reddel, 2010). Although ALT can be found in common cancers, there is a tendency that ALT was found in more tumour types with mesenchymal origin, which is currently still unclear (Lafferty-Whyte et al., 2009). Due to all its unique and important features, telomerase has been intensively studied and has become an attractive anticancer therapeutic target (Harley, 2008) and a potential tumour biomarker for use in oncology.

1.2.3.1 The Telomerase Complex

The telomerase holoenzyme is a ribonucleoprotein that specializes in RNA-dependent reverse transcription (Wyatt et al., 2010). Although the composition of telomerase varies significantly between species (Collins, 2006), it has been found to universally contain a telomerase RNA component (TR or TERC, I use TR for the rest of the thesis); a telomerase reverse transcriptase (TERT) enzyme; and species-specific accessory proteins (Wyatt et al., 2010).

The TR component contains a AAUCCC region complementary to the telomeric repeat sequence (TTAGGG)n, which serves as an RNA template for reverse transcription to elongate the telomere (Feng et al., 1995). This hexameric RNA template sequence is highly conserved in all vertebrates examined so far (Garrido-Ramos et al., 1998, Meyne et al., 1989). In addition to the template sequence, TR also contains secondary structure elements that are essential for the processes of

telomerase accumulation, nuclear localization and the addition of telomere repeats in vertebrates (Theimer and Feigon, 2006). The human TR gene is located at 3q26 chromosome (Sugita et al., 2000) and hTR RNA is first synthesized from 3′-extended precursors transcribed by RNA Polymerase II then processed to a mature hTR mRNA lacking a polyadenosine tail (Collins, 2006). In cancer cells, the expression of hTR is much higher than in normal cells (Yi et al., 1999) and is essential for telomerase activity (Cohen et al., 2007).

TERT is the catalytic subunit of telomerase and is also required for telomerase activity in all species (Nakamura et al., 1997). Experimental evidence has indicated that TERT is the main determinant of telomerase activity (Kanaya et al., 1998), as (1) hTERT mRNA expression levels correlate with telomerase activity (Wu et al., 1999a), and (2) telomerase activity can be restored in telomerase-negative cells by ectopic expression of TERT (Bodnar et al., 1998). HTERT gene is located at the 5p15 chromosome (Bryce et al., 2000).

Various species-specific accessory proteins are also involved in the assembly, accumulation, localization and function of telomerase (Wyatt et al., 2010). Protein components of human telomerase identified so far include the ATPases pontin and reptin (Venteicher et al., 2008), TCAB1 (Telomerase Cajal Body Protein 1) (Venteicher and Artandi, 2009), NHP2 (or NOLA2, Nucleolar Protein Family A, Member 2), NOP10 (or NOLA3, Nucleolar Protein Family A, Member 3) and dyskerin (DKC1) (Fu and Collins, 2007). However, only dyskerin is needed for the catalytic function of active telomerase (Cohen et al., 2007). Dyskerin functions by binding and stabilizing hTR, and also co-operates with NHP2 and NOP10 in the *in vivo* accumulation of hTR (Fu and Collins, 2007).

1.2.3.2 Telomerase Function and Regulation

The active assembled telomerase complex has the ability to copy the same template multiple times, which results in the addition of hundreds of nucleotides to a single DNA primer (Wyatt et al., 2010). During one cycle of telomere elongation, the RNA template is reverse transcribed by TERT which adds nucleotides to the 3’ end of the

telomere, while the lagging-strand synthesis machinery generates the opposite strand (Figure 3). This process is referred to as repeat addition processivity (Autexier and Lue, 2006), and this unique mechanism can rapidly elongate critically short telomeres (Chang et al., 2007).

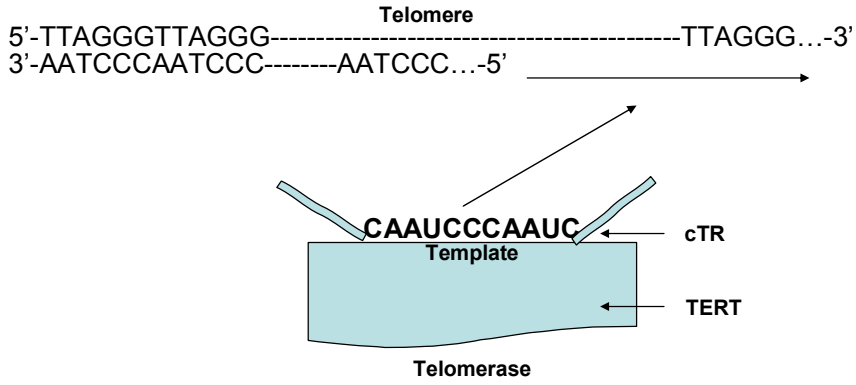


Figure 3 – Telomerase Reverse Transcription - Schematic

Telomerase reverse-transcribes its RNA template into TTAGGG DNA repeats and adds them to the end of chromosome. 3'- CAAUCCCAAUC- 5'can bind to the first few nucleotides of the last telomere sequence and add a new telomeric repeat sequence. It then realigns to the new 3' end of the telomere and repeats the process.

Only TR and TERT can not form the active telomerase (Weinrich et al., 1997), the telomerase ribonucleoprotein complex depends on a series of assembly steps with various other accessory proteins to form the active holoenzyme (Collins, 2006). Telomerase is highly repressed in normal cells by targeting TERT using various transcriptional factors, including Mad (Oh et al., 2000), Menin and SIP1 (Lin and Elledge, 2003) but can also be activated by protein, such as oncogene-encoded c-Myc (Wu et al., 1999b, Cukusic et al., 2008). Estrogen and androgen were also found to upregulate hTERT (Kyo et al., 1999, Misiti et al., 2000, Guo et al., 2003). In addition, alternative splicing of the TERT mRNA occurs in various species, including humans, and is also considered as a mechanism of telomerase regulation (Cong et al., 2002, Kilian et al., 1997).

1.3 Telomerase as a Therapeutic Cancer Target

Since telomerase is specifically upregulated in the majority of cancers, but not in normal somatic cells, it has been widely explored as a promising anticancer target and so far many strategies have been developed to target different components of the telomerase complex (Harley, 2008)(Figure 4).

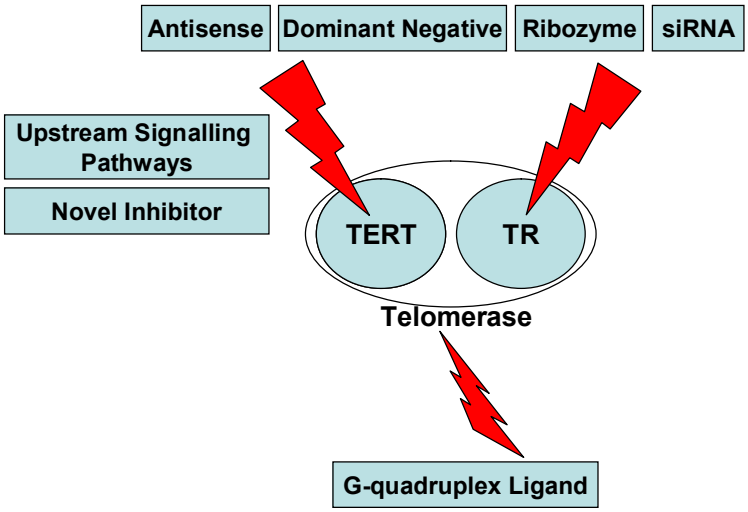


Figure 4 – Major Strategies Targeting Telomerase and its Components

Strategies including antisense, ribozyme and siRNA technology as well as expression of a dominant negative form of either TR or TERT have been used to inhibit telomerase. Some G-quadruplex ligands have also been used to inhibit telomerase in cancer cells. In addition, targeting the upstream signalling pathways of TERT and novel inhibitors have been reported to inhibit telomerase in cancer cells.

1.3.1 Telomerase Inhibition by Targeting TERT

As TERT is the catalytic subunit of telomerase, and as studies have shown TERT to be the main determinant of telomerase activity (Kanaya et al., 1998), it has become a favourite target for many strategies designed to inhibit telomerase function.

For instance, ectopic expression of a dominant-negative TERT protein was found to inhibit telomerase activity in cancerous and non-cancerous telomerase-positive cell lines (Zhang et al., 1999); sensitize cells to chemotherapy agents (Cerone et al.,

2006b, Poynter et al., 2009); and eliminate tumorigenicity *in vivo* (Hahn et al., 1999). Overexpression of a dominant-negative TERT is thought to compete with wild-type TERT for binding to other telomerase components, thus blocking the formation of active telomerase (Nguyen et al., 2009). In support of this, recent evidence has shown that dominant-negative hTERT forms a heterodimer with wild-type hTERT, and that the heterodimer is exported to the cytoplasm for ubiquitin-mediated protein degradation (Nguyen et al., 2009). It is also thought that this heterodimer degradation may be faster than that of the wild-type hTERT homodimer (Nguyen et al., 2009). However, it was also observed that expressing dominant-negative TERT in melanoma cell lines increased their resistance to certain chemotherapeutic drugs (Tentori et al., 2003), and that the long-term expression of dominant-negative hTERT in both human and murine cancer cells could not prevent telomerase reactivation (Delhommeau et al., 2002, Marie-Egyptienne et al., 2008).

Another popular approach for specific gene repression uses ectopic antisense oligonucleotides to bind complementary mRNA targets and to promote target mRNA degradation using an endogenous process (Bennett and Swayze, 2010). Antisense TERT has been shown to target human telomerase to inhibit cell proliferation in various tumour cells *in vitro*, including colon cancer cells (Jiang et al., 2003), liver cancer cells (Du et al., 2003), bladder cancer cells (Kraemer et al., 2003), laryngeal cancer cells (Tao et al., 2005) and pancreatic cancer cells (Wang et al., 2006). Antisense TERT can also sensitize cancer cells to various chemotherapeutic drugs (Yuan and Mi, 2002, Kraemer et al., 2004) and has been effective in inhibiting tumour growth *in vivo* in various human cancer models, such as thyroid cancer (Teng et al., 2003), liver cancer (Lin et al., 2005, Liu et al., 2004b) and cervical cancer (Han et al., 2008). However, despite the current progress made with *in vitro* and *in vivo* experiments, antisense oligonucleotide-based therapy still faces many challenges and the clinical trials have only had limited success so far (Wacheck and Zangemeister-Wittke, 2006).

Ribozyme-based therapy uses special RNA molecules with enzymatic activities to selectively bind to and cleave specific target RNA (Kuwabara et al., 2000). Certain

ribozymes which were originated from the self-splicing group I intron of *Tetrahymena thermophila* can even *trans*-splice an exon attached to its 3' end onto a separate 5' exon RNA (Jones et al., 1996) and have been used to target cancer cells (Shin et al., 2004). These ribozyme-based applications have been shown to target TERT and inhibit telomerase *in vitro* (Yokoyama et al., 2000, Yokoyama et al., 2001, Hayashidani et al., 2005) and also to induce apoptosis in various types of human cancer, including ovarian cancer (Saretzki et al., 2001), breast cancer (Ludwig et al., 2001) and gastric cancer (Hao et al., 2005), as well as in *in vivo* tumour models (Song et al., 2007, Hong et al., 2008, Song et al., 2009).

Other strategies already used to target TERT include novel enzymatic inhibitors and other indirect inhibitory mechanisms. The novel telomerase inhibitor, BIBR1532, is a synthetic non-nucleosidic compound that inhibits telomerase by directly binding to TERT (Pascolo et al., 2002). BIBR1532 has been reported to inhibit telomerase in a variety of human cancer cell lines (Barma et al., 2003, Piotrowska et al., 2005), and this inhibition is associated with telomere shortening and apoptosis (Parsch et al., 2008). BIBR1532 was also observed to sensitize cancer cells to chemotherapeutic drugs (Ward and Autexier, 2005) and appeared to cause a direct cytotoxic effect on malignant cells with higher concentrations of this drug (El-Daly et al., 2005, El Daly and Martens, 2007). On the other hand, however, cancer cells with longer telomeres were resistant to BIBR1532 treatment (Mueller et al., 2007), and further development is necessary to reduce its high IC_{50} value (Barma et al., 2003). In addition, some studies have also shown that TERT can be indirectly activated by epidermal growth factor through the mitogens-activated protein (MAP) kinase signalling pathway (Maida et al., 2002). Inhibitors of the MAP kinase pathway, including Ets and activator protein-1 (AP-1), were found to downregulate TERT transcription (Xu et al., 2007).

1.3.2 Telomerase Inhibition by Targeting the Telomeric G-Quadruplex Structure

The telomeric guanine-rich overhang can be folded into a four-stranded G-quadruplex structure make the 3’-end of the telomere inaccessible and thus unable to

hybridize with the telomerase RNA component, which inhibits telomerase functioning and therefore prevents telomere elongation (Zahler et al., 1991). Since the discovery of telomerase, many quadruplex-binding small ligands have been identified that can stabilize the telomeric G-quadruplex (Neidle, 2010). For example, BRACO-19, 3,6-disubstituted and 3,6,9-trisubstituted acridines and its analogues, have been shown to inhibit telomerase *in vitro* (Fu et al., 2009, Incles et al., 2003) and to have an antitumour effect *in vivo* (Burger et al., 2005, Gunaratnam et al., 2009). A second quadruplex-binding drug, Telomestatin, one of the 2,4,6-triamino-1,3,5-triazine derivatives, has also been reported to inhibit telomerase activity and tumour cell proliferation *in vitro* (Binz et al., 2005), and to inhibit tumour growth *in vivo* (Tauchi et al., 2006). However, mechanisms resistant to such G-quadruplex binders have been reported to exist in primary cancer cells (Sumi et al., 2004), and thus the value of the telomeric G-quadruplex structure as a drug target is still being debated (Wu and Brosh, 2010).

1.3.3 Telomerase Inhibition by Targeting TR

Although many telomerase inhibitors have been discovered using the different strategies mentioned above, very few of the drug candidates have been taken forward for further study, and most of them are still in preclinical development. However, a small telomerase antagonist that targets the RNA component of telomerase is the first telomerase inhibitor to be taken into clinical trial.

GRN163L, or imetelstat, is a short-chain-lipidated oligonucleotide-based therapeutic molecule that binds to the RNA template of telomerase, thus preventing TERT from reverse transcribing the template into telomeric repeats (Roth et al., 2010). This molecule has been tested in various tumour cell lines and in some tumour-initiating cells (Joseph et al., 2010, Marian et al., 2010, Brennan et al., 2010) and has been shown to inhibit telomerase activity, shorten telomeres and reduce cancer cell proliferation *in vitro* (Herbert et al., 2005, Hochreiter et al., 2006, Gellert et al., 2006, Goldblatt et al., 2009b), and to sensitize cancer cells to chemotherapeutic drugs (Djojosubroto et al., 2005, Goldblatt et al., 2009b, Tamakawa et al., 2010, Goldblatt et al., 2009a). GRN163L also has shown anti-tumour activity in several tumour

models *in vivo* (Shammas et al., 2008, Uziel et al., 2010) and is currently in clinical phase I/II trials (Roth et al., 2010).

A few reports have also shown that expression of a dominant negative form of TR can increase levels of apoptosis in some human cancer cell lines (Kim et al., 2001), sensitize tumour cells to anticancer drugs (Cerone et al., 2006a) and exhibit an antitumour effect *in vivo* (Li et al., 2004b).

1.3.4 Telomerase Inhibition Using RNA Interference Technology

Apart from the methods mentioned above, RNA interference technology is another common approach to inhibit telomerase activity by targeting either TR, TERT or other associated proteins (Harley, 2008) and detailed introduction of this section is given in Chapter 3.

1.4 Telomerase as a Biomarker for Cancer

1.4.1 Detection of Telomerase Activity

Since telomerase was identified as a specific and near universal marker of malignancy, the level of telomerase activity has been naturally prompted to be used for cancer diagnosis. The most commonly used method for the detection of telomerase activity is the Telomere Repeat Amplification Protocol (TRAP) (Kim et al., 1994) and its various modifications (Fajkus, 2006). This technique exploits the low substrate specificity of telomerase for targeting a non-telomeric substrate primer (TS). The TS primer is first extended by the telomerase-mediated addition of specific telomeric repeats, and the product is then amplified by PCR using both the TS primer and a reverse primer (Kim et al., 1994). This sensitive TRAP method can detect telomerase activity in samples containing as few as ten cancer cells(Wright et al., 1995), as well as in solid tumour tissue samples and body fluids (Hess and Highsmith, 2002). However, the presence of telomerase activity in some normal cells, especially in proliferative stem cells and activated lymphocytes (Hiyama et al., 1995b), can complicate the interpretation of the TRAP results. Additionally, low telomerase

activity is detectable in certain benign tumours, such as breast fibroadenoma (Hiyama et al., 1996) and thyroid adenoma (Matthews et al., 2001), and this must be considered when analysing data from this assay.

In addition, telomerase activity has also been proposed as a prognostic marker (Hiyama and Hiyama, 2002). Telomerase activity has been found to correlate with poor prognosis for gastric cancer patients, and also with an increased stage of cancer progression (Hiyama et al., 1995a, Tahara et al., 1995). Further, telomerase activity has been identified as an independent predictive factor in the prognosis of colorectal cancer (Tatsumoto et al., 2000).

As telomerase activity requires the expression of both TR template and the TERT protein, the possibility of using their expression levels as prognostic markers has also been tested (Hiyama and Hiyama, 2002). However, hTR and hTERT splice variants can also be detected in cells and tissues that lack telomerase activity (Yi et al., 1999) and (Ulaner et al., 1998). Therefore, caution must be taken when designing specific primers for assaying active hTERT and hTR expression. In conclusion, despite some reports indicating the prognostic potential of telomerase components (Hiyama and Hiyama, 2003), technical problems and the lack of large-scale studies in more than a few cancer types have limited its acceptance so far (Pang and Argyle, 2010).

1.4.2 Circulating TERT mRNA Transcript

Recently, many reports have indicated the diagnostic value of measuring the circulating mRNA transcript levels in human patients with various diseases, especially cancer (Swarup and Rajeswari, 2007). Excitingly, the TERT mRNA transcript was specifically found in serum or plasma samples from various cancer patients, and it may therefore hold promise for cancer diagnosis (Fleischhacker and Schmidt, 2007).

1.4.2.1 The Discovery of Circulating RNA

Although the presence of circulating nucleic acids was first observed in human plasma from both patients and healthy individuals nearly 60 years ago, at that time no hypothesis was proposed to link this observation with any disease (Mandel P, 1948). In fact, circulating DNA was first discovered in human plasma or serum from patients with a variety of disease conditions, such as Systemic Lupus Erythematosus (Tan et al., 1966, Koffler et al., 1973), arthritis (Leon et al., 1981), and in elderly patients with various acute or chronic diseases (Fournie et al., 1993). Following this, elevated levels of circulating DNA were then reported in plasma from various cancer patients compared with healthy subjects (Leon et al., 1977).

In 1999, circulating RNA was reported for the first time in serum or plasma from melanoma patients (Kopreski et al., 1999) and in nasopharyngeal carcinoma patients (Lo et al., 1999). Since then, circulating RNA has been found in the serum or plasma of human patients with various cancers, such as breast cancer (Chen et al., 2000, Silva et al., 2001), lung cancer (Fleischhacker et al., 2001, Schmidt et al., 2005, Sueoka et al., 2005), skin cancer (Hasselmann et al., 2001a, El-Hefnawy et al., 2004), colon cancer (Silva et al., 2002), liver cancer (Miura et al., 2003), prostate cancer (Papadopoulou et al., 2004), oral cancer (Li et al., 2006), nasopharyngeal cancer (Wong et al., 2006) and gastric cancer (Tani et al., 2007).

However, circulating RNA is not only present in cancer patients, but also has been found in other disease and non-disease conditions, such as trauma (Rainer et al., 2004, Bottcher et al., 2006) and pregnancy (Poon et al., 2000, Maron et al., 2007), respectively.

1.4.2.2 The Origin of Circulating RNA

The various different tumour-specific mRNAs found circulating in cancer patients not only reveal the tumour origin, but their discovery is also consistent with many *in vitro* findings that RNA is found in cancer cell culture medium (Morozkin et al., 2004, Bottcher et al., 2006, Valadi et al., 2007). However, normal human blood lymphocytes have also been found to spontaneously release RNA-containing

nucleoprotein complexes *in vitro* in the absence of cell death (Stroun et al., 1978). RNA-containing blebs have also been observed on the surface of thymocytes undergoing spontaneous apoptosis (Biggiogera et al., 1998). Thus, existing evidence suggest the possibility of different sources of circulating RNA.

1.4.2.3 Proposed Existing Forms of Circulating RNA

RNA is much more unstable than DNA and endogenous RNA tends to be degraded by ubiquitous RNases in the blood (Kamm and Smith, 1972). The rapid degradation of exogenous RNA in human plasma samples has been confirmed (Ng et al., 2002, Tsui et al., 2002) plus elevated levels of ribonuclease have also been observed in blood from patients with certain types of cancer (Reddi and Holland, 1976), so it was really surprising when stable circulating RNA was first detected in human plasma or serum (Kopreski et al., 1999, Lo et al., 1999).

Although circulating RNA was discovered relatively recently and its presence has been linked with that of the relatively well-documented circulating DNA in some reports and reviews, there is evidence that circulating RNA and DNA may exist in different forms. For example, a 0.22μm filter captured 95% of plasma RNA, whilst almost all plasma DNA passed through into the filtrate (Ng et al., 2002). Furthermore, an *in vitro* study showed that DNA and RNA were packaged separately during apoptosis (Biggiogera et al., 1998, Halicka et al., 2000), indicating their different biogenesis.

The filtration experiment described above indicates that RNA exists within particles larger than 0.22μm in size, and this could explain why RNA can be stable in the circulation (Ng et al., 2002). Interestingly, adding detergents to plasma resulted in the loss of circulating RNA, which indicates that circulating RNA is likely to be protected in a form of lipid vesicle, or within a protein complex, or possibly in apoptotic bodies (El-Hefnawy et al., 2004).

One of the most popular proposals suggests that circulating RNA is present in tumour-derived, RNA-containing microvesicles that can be found in serum or plasma

of cancer patients (Garcia et al., 2008). Tumour cells have been reported to shed microvesicles, which are fragments of plasma membrane (Taylor et al., 1980). These microvesicles have not only been found in cancer cell cultures (Dolo et al., 1995), but also in the serum or plasma of patients with colorectal (Huber et al., 2005) and oral cancers (Kim et al., 2005). Furthermore, it was found that microvesicles isolated from serum and from cell culture media are very similar (Masella et al., 1989), being characterized as approximately 1μm, circular or villi-like membrane fragments shed by tumour cells (Baj-Krzyworzeka et al., 2006). RNA (Rosi et al., 1988, Baj-Krzyworzeka et al., 2006) and, more importantly, functionally active mRNA has been isolated from these vesicles (Ceccarini et al., 1989, Garcia et al., 2008). The finding of the size of these microvesicles is also consistent with the 0.22μm filter experiment mentioned above.

Another proposed form of RNA-containing particle is the RNA-proteolipid complex that can be found in serum from both patients with malignant disorders, and also in the culture media of malignant cell lines (Wieczorek et al., 1985, Wieczorek et al., 1987). Interestingly, similar RNA-lipid complexes have also been found to be released by human colon carcinoma cells *in vitro* (Rosi et al., 1988).

Furthermore, the RNA-DNA hybrid hypothesis was also suggested to describe a possible form of circulating RNA, and it was demonstrated that adding RNase H to serum reduced the amount of circulating RNA that could be detected (Sisco, 2001). A similar structure was also observed in the cell culture of human lymphocytes (Stroun et al., 1978). However one report indicated that DNA-RNA hybrid is probably not the existing form for circulating RNA as addition of RNase H into the blood samples had no effect on plasma RNA levels (El-Hefnawy et al., 2004).

Although there is no direct evidence that the circulating RNA originates in tumours undergoing necrosis or apoptosis, circulating DNA has been reported to originate from such sources (Jahr et al., 2001). During either spontaneous or DNA damage-induced apoptosis, cellular RNA was observed to be sequestered and packed into apoptotic bodies (Halicka et al., 2000). RNA-containing apoptotic bodies were also

found in melanoma cell culture, and the RNA isolated from cell culture supernatant were protected in human serum in contrast to cell-derived mRNA extracts (Hasselmann et al., 2001). The observation of apoptotic bodies in cancer cell cultures may be explained by their high metabolic rate relative to normal cells (Shaw, 2006). This higher metabolic rate results in a much quicker proliferation rate, characterized by hypoxia and nutrient depletion (Lal et al., 2001, Chaudary and Hill, 2006), and this leads to cancer cell apoptosis.

If circulating RNA does exist in more than one form, the relative contribution of different release mechanisms should be established in order to help researchers to identify the correct RNA population to be used a as tumour marker (Figure 5).

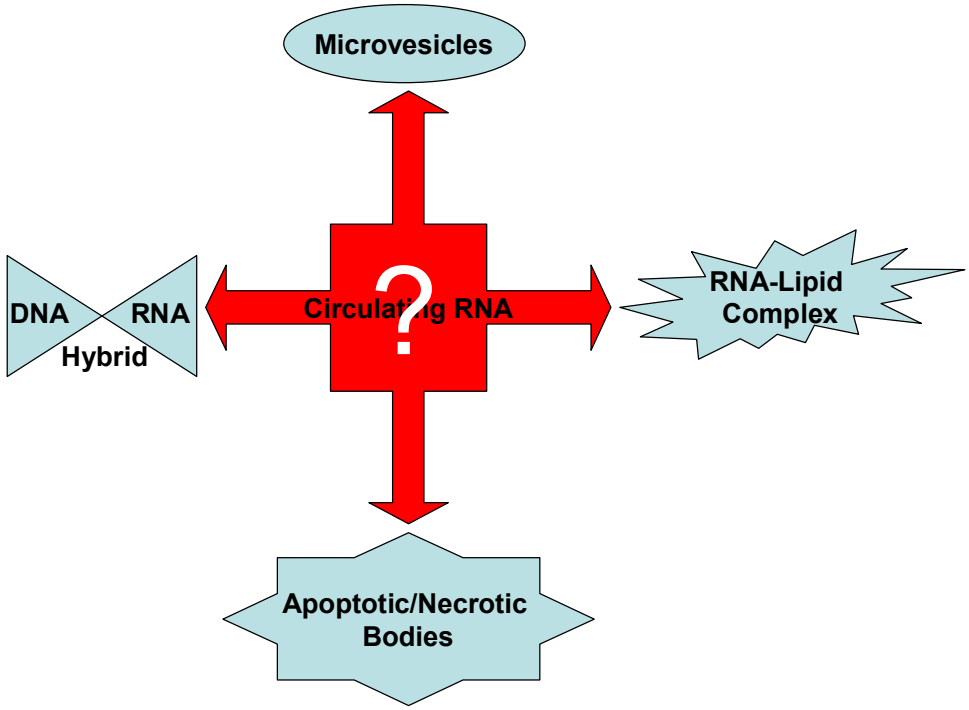


Figure 5 – Proposed Existing Forms of Circulating RNA

The proposed existing forms of circulating RNA include tumour-derived, RNA-containing microvesicles, DNA-RNA hybrid, RNA-lipid complex and RNA-containing apoptotic or necrotic bodies.

1.4.2.4 Active or Passive Release Mechanism of Circulating RNA

Although the proposal that circulating RNA originates from the processes of apoptosis and necrosis indicates that a passive release mechanism may be involved, other evidence suggests that a more active process may exist.

It was observed that the percentage of mRNA in the total RNA extracted from culture media (49.8%) was significantly higher than the percentage of mRNA in RNA extracted from the growing cells (1.4%). This argues in favour of an active release mechanism, as I would expect a similar proportion of mRNA in total RNA extracts from both cells and cell culture media to occur in more passive cell death mechanism (Garcia et al., 2008). It has also been found that the total RNA extracted from plasma contained a large portion of small RNAs that indicates a more active mechanism of circulating RNA may exist (Garcia et al., 2008). Interestingly, tumour cells have been shown to actively promote tumour growth by influencing surrounding stromal cells in the tumour microenvironment (Bhowmick et al., 2004), and tumour-derived mRNA-containing microvesicles have been reported to transfer into monocytes and modulate them to foster tumour growth (Baj-Krzyworzeka et al., 2006, Baj-Krzyworzeka et al., 2007). Tumour-released microvesicles either isolated from cell culture media or from peripheral blood were found to induce Fas-dependent apoptosis in T lymphocytes (Andreola et al., 2002, Huber et al., 2005, Amarzguioui et al., 2005), block the T lymphocyte receptor (Taylor et al., 2003) and promote the differentiation of immunosuppressive myeloid cells that suppress T lymphocyte activity, thus assist in tumour immune escape and contribute to tumour growth (Valenti et al., 2006). It was also found that tumour-released microvesicles can transfer metastatic ability from highly metastatic human tumour cells to poorly metastatic ones (Poste and Nicolson, 1980, Hao et al., 2006) and can also expel cytotoxic drugs through lysosomes and subcellular secretory compartments (Shedden et al., 2003, Safaei et al., 2005).

1.4.2.5 Discovery of Circulating hTERT mRNA

Since the first finding of circulating RNA in serum and plasma from human cancer

patients (Lo et al., 1999, Kopreski et al., 1999), many instances of elevated tumour-specific circulating mRNAs have been linked with different types of cancer, and these observations may lead to a possible early, non-invasive method for cancer diagnostics and prognostics. For example, tyrosinase and other melanoma-specific mRNAs have been detected in the sera of melanoma patients (Kopreski et al., 1999, Rappl et al., 2001). Further, in the sera from lung cancer patients, heterogeneous nuclear ribonucleoprotein B1 (hnRNP-B1) and human epidermal growth factor receptor 2 (Her2/neu) specific mRNAs have been observed (Fleischhacker et al., 2001). The epithelial malignant marker, trophoblast glycoprotein (TPBG or 5T4) mRNA has also been found circulating in breast and lung cancer patients (Kopreski et al., 2001). Interestingly, the circulating epithelial marker carcinoembryonic antigen (CEA) mRNA and also cytokeratin (CK19) mRNA were not only detectable in plasma of colon cancer patients, but were also found to associate with more advanced tumour stages and with circulating tumour cells (Silva et al., 2002). Prostate-specific membrane antigen (PSMA) mRNA was also found in the serum of prostate cancer patients (Chu et al., 2004) and circulating β-catenin mRNA concentration has been found to correlate with tumour stage and tumour removal in the plasma of human colorectal cancer patients (Wong et al., 2004).

In contrast with other tumour markers, hTERT is near universal across cancers; is critical for the oncogenic process of most cancer types; and can also be detected in the early stages of tumorigenesis (Kolquist et al., 1998). Thus many researchers have tried to utilize the hTERT transcript in serum or plasma as an indicator for neoplastic disease. So far many reports have shown that hTERT is not only present in plasma or serum from a variety of cancer patients but also has potential in clinical use.

Circulating hTERT mRNA has been detected in serum or plasma taken from human patients with breast cancer (Chen et al., 2000, Novakovic et al., 2004), follicular lymphoma (Dasi et al., 2001), colon cancer (Lledo et al., 2004), melanoma, thyroid cancer (Novakovic et al., 2004), gastric cancer (Tani et al., 2007) and gynaecological cancer (Miura et al., 2007). In liver cancer patients, circulating hTERT transcript can not only be detected (Waguri et al., 2003), but is also found to independently

correlate with tumour size and the degree of differentiation of the tumour, and is a more sensitive marker than the conventional marker, α-fetoprotein (AFP) (Miura et al., 2003). In lung cancer patients, the circulating hTERT transcript was not only detectable (Pelosi et al., 2006), but its levels were also found to correlate with many clinical parameters, such as tumour size, tumour number, the presence of metastases, recurrence, smoking and surgical treatment, and both the sensitivity and specificity increased when the circulating EGFR transcript was also present (Miura et al., 2006). In prostate cancer patients, circulating hTERT mRNA is not only detectable, but can also be used to distinguish between malignant and non-malignant prostate conditions (Dasi et al., 2006).

Although one group did not find elevated hTERT in their gene profiling experiments to identify circulating RNAs in the plasma of colon cancer patients (Collado et al., 2007), most other findings show a huge interest and enthusiasm for utilizing circulating hTERT transcript measurements in oncology.

1.5 Telomerase in Ageing

1.5.1 Telomerase in Cellular Ageing

Telomerase is not only responsible for tumour immortalization, but has also been linked with ageing (Mather et al., 2011). Unlike cancer cells, which have limitless replicative potential (Hanahan and Weinberg, 2000), normal cells have a finite growth potential, with permanent growth arrest (cellular senescence) being triggered by critically short telomeres or other telomere dysfunctions (Wynford-Thomas, 1999). Tumour cells bypass this cellular ageing barrier by maintaining their telomere length through the activation of telomerase. These findings generated a huge interest in increasing the lifespan of normal somatic cells using telomerase, not only for experimental purposes but also for its relevance to the bigger picture of ageing. The significant ability of cells provided with exogenous telomerase components to bypass replicative senescence and allow unlimited cell proliferation has been reported in several types of human somatic cells (Kang and Park, 2007, Sahin and DePinho, 2010). For instance, the enforced ectopic expression of TERT, the catalytic subunit of

telomerase, has been found to stabilize telomere length in human embryonic kidney cells (Counter et al., 1998), human primary endothelial cells (Baumer et al., 2010), retinal pigment epithelial cells and foreskin fibroblasts, thereby preventing cellular senescence and immortalizing human cells (Bodnar et al., 1998) without generating cell malignant transformation (Jiang et al., 1999).

1.5.2 Telomerase in Stem Cell Functionality

Telomerase not only has an important function in somatic cells, but it is also crucial for stem cell function, as in stem cells telomerase must be constitutively activated to ensure that a lower rate of telomere erosion occurs than in normal cells (Flores and Blasco, 2010). In telomerase-null mice with critically-short telomeres (Flores et al., 2005), the function of epidermal stem cells, as measured by their ability to mobilize and to regenerate skin and hair, is dramatically impaired and this defect can be rescued by the reintroduction of telomerase (Siegl-Cachedenier et al., 2007). At the same time, hyperactivation of telomerase due to elevated TERT expression in a mouse model showed an increased mobilization of stem cells, which resulted in an extended capacity for tissue maintenance associated with an increased lifespan (Donate and Blasco, 2011). The accumulation of stem cells was also observed in telomerase-null mice with critically-short telomeres, which indicates that the stem cells in these mice have an impaired ability to respond to stimuli, and this could explain their premature skin aging phenotype (Flores et al., 2005). In bone marrow, the proliferation (Wong et al., 2003) and regenerative function of haematopoietic stem cells were both found to be impaired in telomerase-null mouse with critically-short telomeres (Rossi et al., 2007), whereas an increased number of haematopoietic stem cells was also observed (Rossi et al., 2008). This effect is not specific to high-turnover tissues, since similar results were also reported for neural stem cells in telomerase-deficient mouse with critically-short telomeres (Ferron et al., 2004, Ferron et al., 2009).

As well as being present in adult stem cells, activated telomerase has also been found in induced pluripotent stem (IPS) cells that exhibit high levels of TERT expression and long telomeres similar to embryonic stem cells (Marion et al., 2009). IPS cells

are generated from differentiated cells by the addition of defined factors, which reprogramme them to become the functional equivalents of embryonic stem cells (Takahashi and Yamanaka, 2006). IPS cells have been generated from both mouse (Okita et al., 2007) and human (Takahashi et al., 2007) cells, and have the capacity to redifferentiate and contribute to a wide range of tissues (Nakagawa et al., 2008), including germlines (Takahashi et al., 2007). Interestingly, the reprogramming efficiency of telomerase-null cells with critically-short telomeres was found to be dramatically reduced, but could be rescued by telomerase reintroduction (Marion et al., 2009).

1.5.3 Telomerase in Ageing

Ageing is fundamentally defined as an overall decline in the function of various organs due to a decreasing ability to maintain tissue homeostasis and thus a decreasing ability to respond to physiological needs and stress (Kirkwood, 2005, Finkel et al., 2007). Ageing in murine and human haematopoietic systems is associated with an age-dependant decline of stem cell function (Rossi et al., 2008), and the ageing of skeletal muscle is accompanied by a decline in the regenerative potential of this tissue (Cerletti et al., 2008). As telomerase plays an important role in stem cell function and prolongs the normal cell lifespan via telomere extension, many studies have investigated its possible role in ageing (Sahin and Depinho, 2010, Espejel et al., 2002). Transgenic telomerase-null mice showed progressive telomere shortening in successive generations, accompanied by a shortening lifespan (Blasco, 2005) and premature age-associated pathologies (Herrera et al., 1999, Espejel et al., 2002), including hypertension (Wong et al., 2009), cardiac dysfunction (Leri et al., 2003) and reduced tissue regeneration. Stem and progenitor cells from various tissues in these mice were found to be either depleted or functionally compromised, possibly due to increased senescence and apoptosis, and to impaired differentiation (Rudolph et al., 1999, Sahin and DePinho, 2010). Further, haematopoietic stem cells from these mice showed a pronounced differentiation profile similar to that found in aged humans (Rossi et al., 2007). Epidermal stem cells from these mice were also found to be impaired in both their proliferative capacity and in their ability to move out of the hair follicle (Flores et al., 2005). Even in the brain, an organ with a

relatively low proliferative activity, those mice exhibited an impaired capacity for neural stem cell renewal and differentiation (Wong et al., 2003). In contrast, transgenic mice with increased telomerase activity due to TERT overexpression were found to have an extended lifespan due to a lower incidence of certain age-related degenerative diseases, mainly those related to kidney function and to germline integrity (Gonzalez-Suarez et al., 2005).

1.5.4 Telomere Length and Ageing

Since the main role of telomerase in cellular ageing, or possibly in organismal ageing, is to control telomere length, the possibility exists that telomere length could be used as an ageing biomarker (Sahin and Depinho, 2010). Mice with short telomeres have been shown to have a reduced lifespan and a reduced stress response (Rudolph et al., 1999). Furthermore, age-dependant telomere attrition has been observed in cells of the lung, pancreas, skin and thyroid of non-human primates (Gardner et al., 2007). In a study of 143 people aged 60 or older, it was found that individuals with shorter telomeres had a significantly higher mortality rate (Cawthon et al., 2003). Three other similar studies also reported the similar results (Bakaysa et al., 2007, Epel et al., 2009, Ehrlenbach et al., 2009). Telomere length has also been found to positively correlate with longevity in Ashkenazi centenarians (Atzmon et al., 2010).

In addition to its effects on lifespan, short telomere length has been associated with several physical ageing parameters such as increased pulse pressure (Benetos et al., 2001) and blood pressure (Mather et al., 2011). In contrast, longer telomere length has been associated with better self-rated general health (Njajou et al., 2009). In addition, telomere length was reported to inversely correlate with levels of psychological stress and the risk of development of psychiatric disease (Epel et al., 2004, Epel et al., 2006, Simon et al., 2006). Patients with inherited degenerative disorders such as dyskeratosis congenita, Werner's syndrome or ataxia telangiectasia, caused by mutations in genes responsible for telomere maintenance (Garcia et al., 2007), have shortened telomeres and reduced lifespans, along with signs of accelerated ageing and bone marrow failure (Kirwan and Dokal, 2009).

Although some reports have suggested that telomere length could be a potential biomarker for ageing, several other studies have failed to find a correlation between telomere length and ageing, lifespan or general health (Martin-Ruiz et al., 2005, Harris et al., 2006, Bischoff et al., 2006, Njajou et al., 2009, Mather et al., 2010). Currently the telomere measurements used in most studies were those from peripheral blood cells, which can be repeatedly sampled with minimum harm to the participant; however, the length of telomeres in these cells may not reflect the length of telomeres in all other tissue types (Thomas et al., 2008). In addition, the current methods for measuring telomere length have their limitations and may not be sensitive enough for detecting very short telomeres (Mather et al., 2011). Taken together, current data regarding the use of telomere length as an ageing biomarker are inconclusive, and it is likely that telomere length may not be a universal biomarker. Instead multiple biomarkers may be required for use at different stages of life to comprehensively evaluate the biological ageing process (Mather et al., 2011).

1.6 Telomerase and Telomere in Other Diseases

In addition to cancer, variations in telomerase activity and telomere length are also associated with a number of other diseases. For example, Cri du chat syndrome (CdCS), also known as chromosome 5p deletion syndrome, is a complex genetic disorder (Cerruti Mainardi, 2006) in which one allele of hTERT is deleted, and the reduced gene dosage is consequently thought to affect telomere maintenance, cell growth and development (Zhang et al., 2003). Further, mutations in the telomerase complex genes, TERT, TR and DKC1 have been reported in both inherited and acquired bone marrow failure syndromes, including dyskeratosis congenita, aplastic anaemia (Yamaguchi et al., 2005) and myelodysplastic syndrome (Knight et al., 2001, Yamaguchi et al., 2003), and these mutations are thought to influence telomere length and thus to attenuate the proliferative potential of haematopoietic stem cells (Yamaguchi, 2007). Recently, telomere biology has also been linked with several cardiovascular diseases, for example shortened telomere length has been reported in patients with atherosclerosis, hypertension and heart failure (Huda et al., 2007, Willeit et al., 2010). However, whether telomere dysfunction is a cause or a consequence of any of these diseases remains to be elucidated (Wong et al., 2009).

Understanding how telomerase functions and how telomere lengths are regulated *in vivo* would not only help us to tackle issues related to cancer, but may also open new areas of ageing research, including ageing-related diseases.

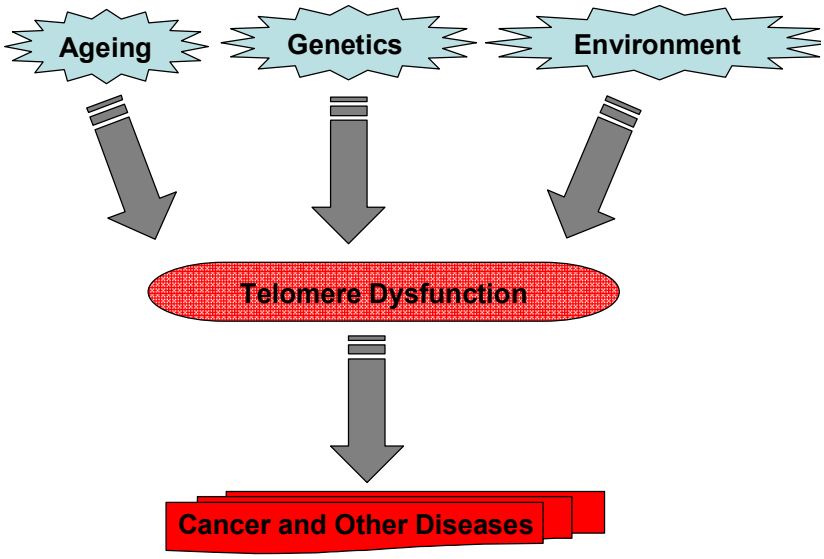


Figure 6 – Current Model between Telomere Dysfunction and Diseases

So far, many evidences have indicated that telomere dysfunction is not only a consequence of ageing but also could be caused by genetic mutations or environmental factors. Dysfunctional telomeres as a result causes many diseases including cancer.

1.7 Canine Telomerase

As occurs in humans, telomerase is also activated in the vast majority of canine cancer patients (Nasir et al., 2001). Currently most *in vivo* models used to study telomere function and telomerase are mouse models. However, the murine system is not ideal, as telomerase is present in all adult mouse tissues and murine telomeres (ranging from 40 to 80 kb) are normally much longer relative to those of humans (Martin-Rivera et al., 1998). Dogs are more similar to humans, in that telomerase activity is upregulated in the majority of tumours, and is absent in normal adult tissues (Argyle and Nasir, 2003). Importantly, canine telomere lengths (3–23 kb, Yazawa et al., 2001, Nasir et al., 2001) are comparable to those found in humans (10–15 kb) (Blasco, 2005). Thus, understanding how canine telomerase functions

may not only benefit canine cancer patients, but may also improve our understanding of human telomerase function and telomere biology (Nasir, 2008).

1.8 Aims of this Thesis

Whilst hundreds of scientific articles on human telomerase and its potential applications in cancer diagnosis are published each year, reports on canine telomerase are very limited so far. In this study, I aimed to answer three fundamental questions regarding canine telomerase. First, we explored the potential of two novel delivery systems and plasmid-based siRNA vector to downregulate cTR gene. We next investigated for the first time if the cTERT mRNA transcript is found in the circulation of canine cancer patients and whether it can be utilized as a cancer biomarker. Finally, in a pilot study I tested whether endogenous telomerase activity can be induced in canine somatic cells by novel telomerase activator compounds. Altogether this study has helped us to gain a better understanding of canine telomerase and its application in canine oncology, and importantly provides a baseline for further investigation.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Cell lines

Cell Lines	Complete Culture Media (500ml)	Origin
293FT	450ml 1X Dulbecco's modified Eagle's medium (D-MEM) high glucose 10% certified foetal bovine serum (FBS; Invitrogen, United States) 0.1mM MEM non-essential amino acids 6mM L-glutamine 1mM MEM sodium pyruvate solution 1% Penicillin-Streptomycin solution (500µg/ml Geneticin®)	Embryonic human kidney cells
EOMA	500ml 1X D-MEM with L-glutamine 10% certified FBS	Murine haemangioendothelioma cells (ATCC-LGC, United States)
SB-HSA	500ml endothelial cell basal medium-2 (EBM-2; Lonza, United Kingdom) EGM-2 SingleQuots (Lonza, United Kingdom)	Canine haemangiosarcoma cells, a gift from Dr. Nasim Akhtar (Akhtar et al., 2004)
HUVEC	The same medium as that used for SB-HSA (described above)	Human umbilical vein endothelial cells (Lonza, United Kingdom)
D-17	450ml 1X D-MEM GlutaMax-1 10% certified FBS 1% Penicillin-Streptomycin solution	Canine osteosarcoma cells (ATCC-LGC, United States)
H1299	450ml RPMI-1640 (Sigma, United Kingdom) 10% certified FBS 1% Penicillin-Streptomycin solution	Human lung cancer cells, a gift from Dr. Matylda Sczaniecka
CEF	450ml 1X D-MEM medium 10% certified FBS 6mM L-glutamine 1mM MEM sodium pyruvate solution 1% Penicillin-Streptomycin solution	Canine embryonic fibroblasts, a gift from my colleague, Daniella Gattegno

Table 2 - Information of all Cell Lines – Names, Origin and Growth Media

All cells and growth media supplements were purchased from Invitrogen, United Kingdom, unless otherwise stated in the table. Growth medium of 293FT cells without Geneticin® was used to recover cells from cryovials and this non-antibiotic medium was replaced with complete medium 24h after culture. EGM-2 SingleQuots which contains growth factors, cytokines and supplements is specially designed to support the growth of primary cells.

2.1.2 General Conditions and Handlings for Cell Culture

All cells mentioned above are adherent cells. 0.25% 1X Trypsin-EDTA, phenol red (Invitrogen, United Kingdom) was used for detaching cells. After 2ml Trypsin-EDTA

was added into the T-75 flask (1ml for T-25 flask; Corning, United Kingdom), cells were incubated at 37°C for 5 minutes and the disassociation of cells was checked by microscope. 1X Sterile Phosphate-Buffered Saline (PBS) which was diluted from 10X PBS (Invitrogen, United Kingdom) contains 1.47mM monobasic potassium phosphate, 137mM sodium chloride, 2.7mM potassium chloride and 4.3mM dibasic sodium phosphate, and was used for washing cells. All cells and related reagents were handled in a sterile laminar flow hood, and reagents were warmed up in 37°C water bath (if necessary) before use. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. All cells were maintained in T-25 or T-75 flasks in either 9ml or 12ml complete growth media respectively. Frozen cell stocks were stored at -70°C (for short term storage) or in liquid nitrogen (for long term storage). All cells were passaged at least three times after thawing before any experiments were done. Cell density, cell viability, β-galactosidase and GFP expression in transfected cells were monitored, analysed and photographed using a fluorescence microscope (Carl Zeiss, Axiovert 40) equipped with Axiovision 4.7 software.

2.1.3 Initiation of Cell Culture

For thawing frozen cell stocks, a T-25 culture flask containing 9ml complete medium was first warmed to 37°C in an incubator. A cryovial containing frozen cells was then thawed quickly in a 37°C water bath, and cells were resuspended in the cryovial and dispensed into the 25cm^2 flask. The flask was gently rocked to evenly distribute the cells, and then it was returned to the incubator. Cells were checked daily until they reached 80–90% confluency for subculture.

2.1.4 Subculture of Cells

When cultured cells reached a confluency of 80-90% they were subcultured. For this, the growth medium was removed and cells were washed twice in 5ml PBS followed by detaching cells using trypsin-EDTA. After cell-detaching, 8ml growth medium was added into the T-75 flask to suspend cells (4ml for T-25 flask). The cell suspension was then transferred into a 15ml sterile conical tube and centrifuged at 150 x g for 5min. After discarding the supernatant, cells were resuspended in 10ml

complete growth medium and counted. The cells were calculated and cell viability was checked using the nigrosin method (see below 2.1.5) before seeding at the required density. For this, cells were diluted into a new T-75 flask with 12ml pre-warmed complete growth medium and checked daily until they were 80–90% confluent. All cells were subcultured in a ratio of 1/3 – 1/8 and growth medium was changed every 2 - 3 days.

2.1.5 Cell Counting and Viability Determination

10µl of cell suspension and 10µl of 0.2% nigrosin (Sigma, United Kingdom) were mixed, and then 10µl of this mixture was added to the counting chamber of a haemocytometer. Cells in the centre 25 squares of the grid were counted (including cells crossing the top and left lines, but not including those touching the bottom and right lines). The blue cells were determined to be dead, and cell viability was monitored to make sure that they were at least 95% viable (the ideal viability for healthy log-phase cultures). The formula below was used for calculating the total number of cells in the cell suspension.

Total No. of cells = cell count x volume of cell suspension (10ml) x 2 (dilution factor with nigrosin) / 0.0001ml (the volume of the 25 squares in the centre of the haemocytometer)

2.1.6 Freezing Cells

After cell culture was established, cells stocks were frozen in cryovials for future use. Each cryovial contained at least 3 x 10^6 viable cells/ml and the freezing medium was 90% complete growth medium, 10% DMSO (Sigma, United Kingdom). For freezing, cells were tryspinised, counted, then centrifuged and resuspended in a pre-determined volume of freezing medium. 1ml Aliquots of cells were dispensed into cryovials. Cryovials were firstly stored at -70°C inside a Styrofoam box for 24 h to slow-freeze cells and then moved to liquid nitrogen for long term storage.

2.2 RT-PCR

All DNA oligonucleotides used in this study (including PCR primers, sequencing primers and siRNA inserts) were ordered from Eurofins MWG Operon. All sequencing was performed at the GenePool sequencing service at the University of Edinburgh using Sanger Sequencing method.

2.2.1 Total RNA Isolation

Total cellular RNA was extracted using an RNeasy Mini Kit (Qiagen, United Kingdom). All components mentioned below were provided by the kit unless otherwise stated. Up to 1 x 10^7 cells can be processed in one reaction. Cells were sedimented by centrifugation at 300 x g for 5min and the supernatant was carefully removed. 600µl RLT buffer was then added and cells were disrupted by pipetting. The cell lysate was loaded into a QIAshredder spin column on top of a collection tube and centrifuged at full speed for 2min. After lysing cells, 600µl of 70% ethanol was added to the homogenized lysate. Up to 700µl samples were loaded into the RNeasy Spin Column assembly and centrifuged at 8,000 x g for 15s. After discarding the flow-through, 700µl RW1 buffer was added and the column was centrifuged at 8,000 x g for 15s to wash the membrane. After discarding the flow-through, 500µl RPE buffer was added and the column was centrifuged at 8,000 x g for 15s. This last step was then repeated, but with a 2min centrifugation step. After discarding the flow-through, RNeasy spin column was placed into a new tube and centrifuged at full speed for 1min to spin out any buffer left in the column. Following this, the RNeasy spin column was transferred to a new collection tube and 40µl Nuclease-free water (NF-water) was added to the membrane. The column was then centrifuged at 8,000 x g for 1min, and then the eluted RNA was treated with DNase before being used for experimental applications.

2.2.2 DNase Treatment

DNA-*free*™ kit was used for all DNase treatment of RNA extracts. All components mentioned below were provided by the kit unless otherwise stated. 0.1 volume of 10X DNase I buffer and 1.5µl DNase I was added to 40µl RNA in a 500µl

microcentrifuge tube, mixed gently then incubated for 30min at 37ºC. Next, 0.1 volume of DNase Inactivation Reagent was added and mixed. After incubation for 2min at room temperature, the tube was centrifuged at 10,000 x g for 1.5min and RNA was then transferred to a fresh tube and quantified by spectrophotometry.

2.2.3 Spectrophotometry

The concentrations of DNA or RNA samples were quantified using a NanoDrop™ Spectrophotometer (Thermo, United States). Before measuring samples, 2µl NF-water was applied to the machine to make a blank measurement. 2µl of DNA or RNA was then applied to the machine. The ratio of the absorbance readings at 260nm and at 280nm ($A_{260/280}$) was calculated automatically by the machine and used to estimate the purity of DNA and RNA samples. For pure DNA the ratio is close to 1.8 and for pure RNA the ratio is close to 2.0.

2.2.4 cDNA Synthesis Using Cellular RNA

All cDNA synthesis from cellular RNA extracts was done using the Reverse Transcription System (Promega, United Kingdom), and synthesized cDNA was used in conventional and real-time PCR reactions. The recipe for the reaction mix is given below (Table 3). All components mentioned below were provided by the kit unless otherwise stated. The reaction mixture was first incubated at room temperature for 10min, and then the reverse transcription reaction was done at 42ºC for 15min. The reaction products were then heated to 95˚C for 5min to inactive the transcriptase and prevent it from binding to the cDNA, then cooled at 4˚C for 5min. cDNA can be stored at -20˚C.

Component	Amount (µl)
$MgCl_2$, 25mM	4
Reverse Transcription 10X Buffer	2
dNTP Mixture, 10mM	2
Recombinant RNasin® Ribonuclease Inhibitor	0.5
AMV Reverse Transcriptase, 15U	0.8
Random Primers, 0.5µg/µl	1
Total RNA	1µg
NF-water	To a final volume of 20

Table 3 – cDNA Synthesis from Cellular RNA

This cDNA synthesis kit was used on all cellular RNA extracts only. For serum RNA, cDNA synthesis was done using Sensiscript RT kit detailed in Chapter 4 (4.3.3).

2.2.5 RT-PCR

RT-PCR methods and conditions for different applications are provided in the appropriate results chapter.

2.2.6 Agarose Gel Electrophoresis

2% agarose gels were used to visualize restriction digestion products of 55bp in size. It was prepared using 0.7g agarose (Sigma, United Kingdom) and 35ml 1X TAE buffer. 1X TAE buffer was prepared by diluted 50X TAE buffer (Qiagen, United Kingdom) and contains 40mM Tris acetate and 1mM EDTA. Larger PCR products were visualized using 1% agarose gels, which were prepared using 0.35g agarose and 35ml 1X TAE buffer (for a small gel) or 1.2g agarose gel and 120ml 1X TAE buffer (for a large gel). The agarose was weighed and added to the correct volume of TAE buffer, then heated in the microwave at full power for 30s at a time until the agarose had melted. When the bottle was cool enough to handle, 3.5µl (for a small gel) or 12µl (for a large gel) of GelRed™ (Biotium, United Kingdom) was added and the molten agarose was then poured into a gel tray and allowed to set for at least 1 h. DNA samples were mixed with Blue/Orange Loading Dye (Promega, United Kingdom) at a ratio of 1:5 v/v. For cell experiments, 10µl PCR product samples were analysed, and for serum RNA experiment, 25µl PCR product samples were analysed. Electrophoresis was done in 1X TAE buffer, and either 100bp or 1kb DNA ladder

(Promega, United Kingdom) were run alongside samples. Gels were run at either 60V (for a small gel) or 120V (for a big gel) for approximately 40min. After electrophoresis, gels were visualised and analysed on Gel Doc XR+ System with Quantity One software (Version 4.6.8).

2.2.7 Transformation of *E. coli*

LB plates and LB broth were first prepared before transformation of *E.coli*. LB plates were prepared using imMedia™ Amp Blue premixed powder (Invitrogen, United Kingdom). A single sachet was mixed with 200ml distilled water then heated in a microwave oven on a medium power setting for 2–3min until bubbles started to appear, then heating was continued for another 30s reheat until the powder was completely dissolved. Every 10ml molten medium was then gently poured into standard-sized sterile Petri dishes to reach 1/2 full at most. These plates were allowed to cool down, then sealed and put upside down in the refrigerator for up to a month until use. LB broth was prepared by mixing 25g Luria Broth Base (Invitrogen, United Kingdom) with 1L distilled water and autoclaving. Ampicillin (Sigma, United Kingdom) was added to cooled LB broth for a final concentration of 100µg/ml.

One Shot TOP10 competent *E. coli* cells (Invitrogen, United Kingdom) were transformed with plasmid DNA. 2µl DNA was first added to a thawed vial of 50µl competent cells, and mixed. After incubating for 5min on ice, cells were heat-shocked for 30s at 42˚C in a water bath then immediately transferred onto ice. 250µl super optimal broth with catabolite repression (S.O.C.) medium (room temperature) was added to the cells, then the vial was shaken at 200rpm in a 37˚C incubator for 1h. 20µl of transformed cells were spread onto a pre-warmed LB plate and incubated overnight at 37˚C. The next day, transformed bacterial colonies were picked and grown on for plasmid preparation.

2.2.8 Plasmid DNA Preparation

Two methods were used to prepare plasmid DNA: the QIAprep Miniprep Kit (Qiagen, United Kingdom) was used for small-scale DNA isolation to screen

colonies, and the Hispeed Plasmid Maxi Kit (Qiagen, United Kingdom) was used for large-scale DNA isolation. All the buffers and reagents used below were supplied in the kits unless otherwise stated. The manufacturer's manuals were followed when using both kits.

2.2.8.1 Plasmid Preparation Mini Kit

A single colony was picked from a plate and used to inoculate 3ml LB medium containing 100µg/ml ampicillin. After 14h incubation at 300rpm in a 37˚C shaking incubator, bacteria were harvested by centrifugation at 6800 x g for 3min at room temperature. The bacterial pellet was resuspended in 250µl P1 buffer and transferred to a microcentrifuge tube. 250µl P2 buffer was then added and the tube was inverted 6 times to mix. 350µl P3 buffer was then added and mixed immediately by inverting the tube another 6 times. The tube was then centrifuged for 10min at 17,900 x g at room temperature. The supernatant was then decanted into a QIAprep spin column, which was then centrifuged for 1min. After discarding the flow-through, 0.5ml BP buffer was added and the column was centrifuged for 1min and the flow-through discarded. The column was then centrifuged for 1min again to remove residual wash buffer. Lastly, the column was placed in a 1.5ml microcentrifuge tube and 50µl EB buffer was added to the centre of the membrane. After 1min incubation, the column was centrifuged for 1min and plasmid DNA was eluted into the microcentrifuge tube.

2.2.8.2 Plasmid Preparation Maxi Kit

For the Hispeed Plasmid Maxi Kit, a single bacterial colony was picked and inoculated into 5ml LB medium containing 100µg/ml ampicillin to form a starter culture. This was shaken at 300rpm for 8h at 37˚C. Following this, 0.6ml of the starter culture was diluted 1/500 with 300ml LB containing 100µg/ml ampicillin in a 1L flask and shaken at 300rpm for 14h. Following this, the bacteria were harvested by centrifugation at 6,000 x g for 15min at 4˚C. The bacterial pellet was resuspended in 10ml chilled P1 buffer for lysis, then 10ml P2 buffer was added and thoroughly mixed. After no more than 5min incubation at room temperature, 10ml chilled P3 buffer was added and mixed by inverting 6 times, then the lysate was immediately

poured into the barrel of a QIAfilter cartridge. The plunger was then inserted into the cartridge and cell lysate was filtered through a Hispeed Maxi Tip that had been previously equilibrated with 10ml QBT buffer. After the cleared lysate had passed through the resin by gravity flow, the tip was washed with 60ml QC buffer. Then the DNA was eluted from the tip using 15ml QF buffer. 10.5ml (0.7 volumes) room temperature isopropanol was then added to the eluted DNA, mixed and incubated for 5min at room temperature. After this incubation, the eluted DNA/isopropanol mixture was transferred to a 30ml syringe attached to a QIAprecipitator Maxi Module, and the mixture was pressed through the precipitator using a plunger. The precipitator was then removed followed by removal of the plunger. After reattaching the precipitator, 2ml 70% ethanol was added into the syringe to wash the DNA and the plunger was inserted to press the ethanol through using constant force. Next air was pressed through the syringe quickly and forcefully to dry the membrane. This step was repeated, and then the nozzle of the precipitator was dried with absorbent paper to prevent any ethanol carryover. The precipitator was attached to a new 5ml syringe held over a 1.5ml collection tube. 1ml of TE buffer was added to the membrane through the syringe and DNA was eluted into the collection tube using constant pressure. The eluate was then transferred back in to the syringe assembly and a second elution was performed.

The concentration of purified DNA was measured using a NanoDrop machine, and DNA was stored at -20˚C.

CHAPTER 3

EXPLORATION OF NOVEL SYSTEMIC DELIVERY SYSTEMS FOR PLASMID-BASED siRNA VECTOR TARGETING cANINE TELOMERASE

3.1 Chapter Abstract

Although we have developed a siRNA viral vector to target canine telomerase *in vitro*, an efficient systemic delivery vehicle is still required. Some endothelial lineage cells have been reported to specifically home to the tumour formation sites and a novel dendrimer, PPIG3 was also found to accumulate in tumour sites following i.v. injection. I therefore explored these novel delivery systems in the canine context. In addition, I developed a plasmid-based siRNA vector targeting cTR and tested it on two canine cancer cell lines.

A series of transient and stable transfections were conducted on 3 endothelial cell lines using 3 different reporter genes by 4 different commercial transfection reagents. After succeeding the transient and stable transfection on SB-HSA cells, I labelled them and analyzed their tissue distribution into a canine xenograft tumour mouse model after i.v. injection. In parallel, the transfection ability of PPIG3 dendrimers was also tested on two canine cancer cell lines. To this end, I then designed and constructed a plasmid-based siRNA vector and tested its ability to downregulate cTR in two canine tumour cell lines by RT-PCR.

Initially, I have found SB-HSA cells were more easily transfected than the other two cell lines and by using a modified protocol of Lipofectamine™2000, I could even achieve better transient and stable transfection results on SB-HSA cells. *In vivo* however, although I did observe some labelled SB-HSA cells homing to tumour sites, a wide non-specific tissue distribution was also observed. In parallel, PPIG3 dendrimers-mediated DNA transfer *in vitro* was found not as effective as using Lipofectamine™2000 method. Using plasmid-based siRNA vector, I did not observe any cTR gene-silencing on two canine cancer cell lines.

Although our potential cellular delivery vehicle, SB-HSA cells was optimised *in vitro*, their tumour-homing ability as well as the wide tissue distribution still needs further investigation. For PPIG3 dendrimers, their relatively low transfection on canine cells may hinder their performance as a systemic delivery vehicle *in vivo* and so this system also needs further development. Since our siRNA vector did not show any

cTR gene-silencing, it has raised the question to whether the cTR gene sequence I targeted is a valid target for siRNA as well as of whether the siRNA vector itself is capable of inducing RNAi in canine cells.

3.2 Introduction

Despite recent progress in cancer treatment, cancer gene therapy use is limited with disappointing results so far. The current inefficiency of cancer gene therapy is caused by many factors, including the low efficiency of gene transfer; the immune attack and destruction of therapeutic molecules or the delivery vehicle; non-specific targeting of cancer cells; insufficient delivery of therapeutic molecules to the target site; and delivery approaches that restrict the delivery of therapeutic molecules to the local tumour site only. One of the most important factors is the lack of a systemic delivery system, which would ideally deliver the therapeutic gene to, and thereby mediate transgene expression in, different tumour-cell populations and in all primary and metastatic tumour sites.

3.2.1 Systemic Delivery Vehicles for Cancer Gene Therapy

Naked ssDNA oligonucleotides have a half-life less than an hour in peripheral blood, even when modified to resist degradation (Iversen, 1991), and are rapidly cleared from the kidney (Sands et al., 1994). Naked dsDNA is also cleared rapidly after being administered into the blood circulation and is extensively taken up by the liver (Kawabata et al., 1995). In addition, siRNA molecules cannot resist degradation from blood and are subject to a similar elimination pathway following intravenous injection (Huang et al., 2011). Furthermore, cellular uptake of both DNA and RNA is generally inefficient. All of these factors strongly hinder delivery of the therapeutic gene to tumour sites. To assist the systemic delivery of therapeutic genes, several promising delivery systems are now being proposed and investigated: these are generally classified into viral vectors (Sinkovics and Horvath, 2008) and non-viral vectors (Roth et al., 2008, Kaneda, 2010) (Table 4).

Systemic Delivery Vehicles							
Viral Vectors	Non-Viral Vectors						
	Synthetic Vectors			Biological Vectors			
	Liposomes	Polymers		Bacterial Vectors		Cellular Vehicles	
		PEI	Dendrimers	Obligate Anaerobic Bacterial Vectors	Facultative Anaerobic Bacterial Vectors	MSC-based Vehicles	Endothelial Lineage Cell-based Vehicles

Table 4 – Summary of the Major Current Systemic Delivery Vehicles

Current systemic delivery vehicles are classed into viral vectors and non-viral vectors. Non-viral vectors include synthetic vectors and biological vectors. Synthetic vectors mainly include liposomes and polymers among which polyethylenimine (PEI) and other dendrimers are popular candidates that are currently being explored. Among biological vectors, bacterial vectors and cellular vehicles have been intensively studied. Currently both anaerobic and facultative anaerobic bacteria have been investigated as potential systemic delivery vehicles. For cellular vehicles, mesenchymal stem cells (MSC) and endothelial lineage cells have been proposed as candidates for systemic gene transfer vectors.

3.2.1.1 Viral Vectors

Viruses appear to be an attractive delivery vehicle because they contain intrinsic mechanisms designed to transfer their DNA into host cells (Baranowski et al., 2001). Part or all of the viral genome can be modified by replacement with therapeutic genes, which enables the virus to be engineered into either a replication-defective or a replication-competent recombinant viral vector (Kay et al., 2001). To date, several types of viral vector have been developed for cancer gene therapy, including retroviral vectors, adenoviral vectors and herpes viral vectors, and these have been shown to be efficient gene transfer vectors for both *in vitro* and *in vivo* applications (Sinkovics and Horvath, 2008).

Retroviral Vector

The retrovirus is a single-stranded RNA (ssRNA) virus encoding a reverse transcriptase enzyme that can produce DNA from its RNA genome, and then insert the DNA into the host genome in order to replicate itself. Thus, the major advantage of this vector is its ability to induce long-term gene integration into the host genome,

raising the possibility that transferred therapeutic genes could be expressed throughout the entire life of the host cell (Dalba et al., 2007). Early clinical trials on replication-defective retrovirus-mediated cancer gene therapy indicated a low transduction rate for tumour cells *in vivo*, thus paving the way for using replication-competent vectors in a clinical setting (Rainov and Ren, 2003). Indeed, intratumoural injection of a replication-competent murine leukaemia virus carrying a suicide gene into a cancer-bearing mouse resulted in a 100% survival rate (Wang et al., 2003). In addition, a promising member of the retroviral family, the lentivirus, has been developed as a siRNA expression vector, and has been shown to effectively deliver siRNA and induce post-transcriptional gene-silencing *in vitro* and *in vivo* in various cancer models (Sumimoto and Kawakami, 2007). Additionally, unlike other retroviral vectors, the lentivirus has the ability to transduce non-dividing cells (Bukrinsky et al., 1993) which has become an attractive candidate to treat brain cancer.

Adenoviral Vectors

An adenoviral vector has also been developed for cancer gene therapy (Fukazawa et al., 2010). The adenovirus has been shown to transfer foreign genes into a broad range of cells, including both dividing and non-dividing cells (Shirakawa, 2008). First generation adenoviral vectors were also reported to induce an unfavourable host cellular immune response (Yang et al., 1995), and therefore more viral genes have been deleted in new generation adenoviral vectors and these exhibit less immunogenicity and have the ability to mediate long-term gene expression in host cells (Sakhuja et al., 2003). This vector requires a helper virus, which has been rendered unpackable in the host cells but retains the ability to remain replicate and provide helper function, to be fully functional thus should have increased transduction ability, increased safety and reduced immunogenicity (Parks et al., 1996). It is currently one of the most promising viral vectors and more research is still required to improve cancer-cell targeting as well as transduction efficiency (Fukazawa et al., 2010).

Herpes Simplex Viral Vector

The herpes simplex virus (HSV) is a neurotropic virus known for its ability to infect nerve cells and cause latent infection, and HSV-1 has been the serotype most studied in cancer gene therapy applications (Marconi et al., 2008). Similar to lentiviral vectors, HSV also has the ability to infect both dividing and non-dividing cells and can grow robustly inside a tumour (Todo, 2002, Todo, 2008). The early generation replication-defective HSV vectors were developed to deliver transgenes into tumour cells such as melanoma (Krisky et al., 1998) and glioblastoma (Niranjan et al., 2000). One type of HSV-1 vector, the amplicon vector, is identical to wild type HSV but carries a DNA plasmid instead of the viral genome (Sena-Esteves et al., 2000), and has been utilised to induce RNAi to silence crucial cancer genes, such as EGFR, in human gliomas (Saydam et al., 2005). Various replication-competent HSV vectors have also been engineered to not only target cancer cells, but have also been developed as oncolytic vectors that retain the ability to lyse and kill the host cell, thereby releasing new virus particles capable of infecting neighbouring tumour cells (Marconi et al., 2008). However, some clinical trials conducted using the HSV vector did not show significant tumour growth retardation (Rampling et al., 2000, Markert et al., 2000). Overall, although some progress has been made in the use of HSV viral vectors, a combinatorial approach using viral vectors along with chemotherapy might achieve a better result for treating cancers (Post et al., 2004).

However, the major concerns involving the use of viral vector include safety concerns; immunogenicity; insertional mutagenesis that may cause oncogene activation and silencing of tumour suppressor genes. The extent of these concerns indicates that further development of viral vectors for use as cancer therapeutics is still needed (Tomanin and Scarpa, 2004).

3.2.1.2 Non-viral Vectors

Synthetic Non-viral Vectors

In general, non-viral vectors can be classified into synthetic vectors and biological non-viral vectors. Currently synthetic vectors are mainly produced using chemical means, and include cationic liposomes and polymers that can self-assemble with the

negatively-charged nucleic acid to form DNA–vector complexes (or lipoplex) (Kaneda, 2010). It is the most common method employed for the 'packaging' of nucleic acid which is based on electrostatic interaction between the anionic nucleic acid and the positive-charged synthetic to form the nanoparticles where nucleic acid is complexed and condensed. Apart from delivery to the desired site, this packaging nanoparticle also tends to compact and protect the nucleic acid (Brown et al., 2001). Over the past decade, increased interest has been shown in the use of synthetic vectors as delivery vehicles for cancer gene therapy, not only because they can deliver therapeutic DNA or RNA molecules but also because they can deliver chemotherapy drugs to tumour cells, protect these drugs while circulating in the blood and also because they have a lower immunogenicity than viral vectors (Morille et al., 2008).

Liposomes

Cationic liposomes are amphiphilic molecules that can spontaneously form a lipid bilayer in aqueous solution. They were first introduced to facilitate gene transfer *in vitro* (Felgner et al., 1987) and soon became popular as non-viral transfection methods (Tarahovsky, 2009). Currently, the internalization of lipoplexes by cells is still not well understood, but early reports suggest that fusion with the cellular plasma membrane results in DNA delivery direct to the cytosol. However, some endocytosis blocking studies have shown that most of the uptake occurs via endocytosis (Pichon et al., 2010). Some research has demonstrated that lipoplex of size 100–120nm had the best tumour uptake result (Moreira et al., 2001). Intratumoural lipoplex-mediated cancer gene therapy has been demonstrated in melanoma patients (Nabel et al., 1993), and the systemic delivery of immuno-siRNA–lipoplex with specific tumour targeting has also been shown to be effective in reaching both primary and metastatic tumours in three different tumour models (Pirollo et al., 2006) and in several other studies (Wu and McMillan, 2009). Although many liposomes have been synthesized for cancer gene therapy (Templeton, 2009), very few of them have reached clinical trials so far. The limitations of using liposomes as systemic delivery vehicles include aggregation and disintegration of the vector in the blood circulation (Li et al., 1999); non-specific liver uptake mainly by

Kupffer cells and consequent inflammatory side-effects (Zhang et al., 2005, Tousignant et al., 2000); and relatively low transfection efficiency compared with viral vectors (Tong et al., 2009).

Polymers

Polymers are large molecules containing repeating structural units and were reported as gene transfer vectors *in vitro* at approximately the same time as liposomes (Wu and Wu, 1987).

PEI

The most common non-degradable polymer used for DNA transfer is polyethylenimine (PEI) (Boussif et al., 1995), which exists in both linear and branched forms and has a high level of gene transfer efficiency (Halama et al., 2009). PEI is water soluble and consists of multiple ethylenimine units that contain a high density of positive charges contributed by their amino groups (Park et al., 2006). PEI was found to mediate gene transfer with a comparable efficiency to those of viral vectors *in vitro* (Kichler et al., 2001), and this transfection efficiency is associated with its high affinity for negatively-charged cells that assists the polymer–DNA complex (polyplex) uptake (Halama et al., 2009). The exact mechanism of polyplex uptake by cells still remains to be elucidated, but the structure of PEI was found to assist endosome escape by destabilizing the organelle membrane and cause DNA release to the cytosol (Kichler, 2004). Several reports have demonstrated that the systemic administration of tumour-specific targeting-ligand-coated polyplexes could deliver transgenes to tumour sites (Kircheis et al., 2001, Hildebrandt et al., 2003, Kursa et al., 2003), and effective therapeutic effects of PEI-mediated delivery of therapeutic plasmid DNAs (Xu et al., 1999), antisense oligonucleotides (Rait et al., 2002) or siRNA molecules (Hu-Lieskovan et al., 2005) were also observed in several mouse tumour models. However PEI-based cancer gene therapy also face challenges, such as non-specific lung, liver and spleen uptake and their induction of inflammatory side-effects, and these problems indicate the importance of considering such factors as structure and molecular weight when designing polymer vectors

(Jeong et al., 2007, Bonnet et al., 2008).

Dendrimers

Polypropylenimine (PPI) dendrimers are synthetic polymers with a core structure, and symmetric branches (Tomalia et al., 1985), and their efficient gene transfer ability was first shown in 1997 (Delong et al., 1997). Since then the gene transfer ability of numerous dendrimers has been studied, and their unique structure has been shown to provide a positively charged surface that can form complexes with negatively charged nucleic acids (Guillot-Nieckowski et al., 2007). When complexed with DNA, dendrimers were found to spontaneously form nanoparticles that are colloidal stable without any flocculation or aggregation and are well-organized nanostructures that DNA could condense into (Chisholm et al., 2009).

Generally, the higher the charge density (the generation) of the dendrimer, the higher the transfection efficiency (Dufes et al., 2005). However, higher generation dendrimers are associated with higher toxicity (Zinselmeyer et al., 2002), although the general cytotoxicity of dendrimers is still considered to be lower than some other polymers, such as PEI (Fischer et al., 2003). It was also reported that the transfection capability of dendrimers was at least comparable with that of other cationic polymers (Gebhart and Kabanov, 2001). Dendrimer-mediated gene transfer in cells is thought to utilise their pH buffering capacity, which could facilitate release of the bound nucleic acid from the DNA–dendrimer complex in cells (Sonawane et al., 2003).

PPI dendrimers consist of a butylenediamine (DAB) core with branched units that expand by Michael addition of acrylonitrile to a primary amino group followed by hydrogenation of nitrile groups (Dufes et al., 2005), and which can now be synthesized easily (de Brabander-van den Berg and Meijer, 1993). PPI dendrimers have been developed commercially as transfection agents, and their different generations have been reported to efficiently transfer genes and to have a positive toxicity profile *in vitro* (Agashe et al., 2006) and *in vivo* (Tack et al., 2006). Indeed, some dendrimers have been shown to be more efficient gene transfer reagents than

PEI (Kim et al., 2007). Several recent studies using PPI dendrimers, especially PPI generation 3 (PPIG3), have demonstrated their potential in cancer gene therapy (Tekade et al., 2009).

It has been reported that of all the generations of PPI, PPIG3 has the highest transfection efficiency combined with adequate cytotoxicity on an epithelial carcinoma cancer cell line (Zinselmeyer et al., 2002). Similar findings were also reported for several other cancer cells (Hollins et al., 2004, Santhakumaran et al., 2004). For example, injection of PPIG3 complexed with a TNF-α-expression plasmid into the tail vein results in the efficient accumulation of TNF-α in tumour sites in murine subcutaneous tumour models, and induces tumour necrosis and interestingly injection of PPIG3 alone also showed some anti-tumour activity (Dufes et al., 2005a). Further, intravenous administration of oligoethylenimine-grafted PPIG2 and PPIG3 resulted in an accumulation of both molecules in subcutaneous tumours, with PPIG3 being accumulated more efficiently (Russ et al., 2008). In addition, the intravenous administration of transferrin-conjugated PPIG3, whose receptors are often overexpressed in numerous cancers, resulted in its selective accumulation in tumour sites, the induction of tumour regression and led to 100% long-term survival with no signs of toxicity (Koppu et al., 2010). In a recent *in vivo* imaging study, PPIG3 showed highly specific tumour targeting of tumour-bearing mice, with no detection of gene transfer occurring in any of the other tissues, and this was thought to be achieved by the enhanced permeability and retention (EPR) effect of nanoparticles that formed with PPIG3 and DNA (Chisholm et al., 2009). The EPR effect constitutes an important mechanism by which certain objects especially with high molecular weight drugs can extravasate through the endothelial gaps of tumour blood vessels, which are usually larger and more disorganised than normal blood vessels (Matsumura and Maeda, 1986, Iyer et al., 2006).

Biological Non-viral Vectors

Besides viral and synthetic vectors, certain bacteria (Gardlik and Fruehauf, 2010) and mammalian cells (Roth et al., 2008) have also been discovered to be potential delivery vehicles for cancer gene therapy.

Bacteria-based vectors

The recent advances in cancer gene therapy and the findings that hypoxic and necrotic regions are consistently present within solid tumours (Thomlinson and Gray, 1955) have prompted the notion of using genetically altered anaerobic bacteria as delivery vehicles. Hypoxia has been found having a pivotal role in tumour angiogenesis, development and metastasis (Zhou et al., 2006). Although lower transfection efficiencies have been observed in hypoxic cells (Dachs et al., 2000), the hypoxic regions in tumours provide an ideal place for anaerobic bacteria to grow, thus providing an excellent opportunity for the development of bacteria-based delivery vehicles. In phagocytic cell gene transfer, bacteria can be transported into cells in phagosomes, then escape from the phagosome and replicate in the cytosol (Grillot-Courvalin et al., 2002). In non-phagocytic cells, bacteria-mediated gene transfer can occur through the normal routes of pathogen entry to cells (Marra and Isberg, 1996), and recombinant bacteria have been reported to transfer synthesized protein (instead of DNA) to target cells as a consequence of the substantial level of activity of eukaryotic promoters driving expression of the transferred genes (Goussard et al., 2003). Due to these advantages, combined with their preference for tumour regions remote from vasculature where synthetic vectors can only travel passively, anaerobic bacteria have become regarded as a better delivery vehicle for cancer gene therapy (Vassaux et al., 2006). Since the first study of bacteria-based gene transfer (Sizemore et al., 1995), there have been many reports regarding bacteria-based cancer gene therapy (Jia and Hua, 2009). Predominantly, four types of attenuated anaerobic bacteria have been studied for cancer gene therapy so far: *Bifidobacteria* and *Clostridia* (anaerobes) and *Salmonella* and *Escherichia* (facultative anaerobes) (Forbes, 2010).

Obligate Anaerobic Bacteria-based Vectors

Obligate anaerobes such as *Clostridia* and *Bifidobacteria* cannot survive in oxygen, and bacterial spores injected into tumour should only germinate in anoxic regions.

Clostridia-based Vector

The specific tumour colonization of *Clostridia* after intravenous administration of the spores was reported nearly 60 years ago (Malmgren and Flanigan, 1955). After screening of 26 common anaerobic bacteria, an attenuated strain, *Clostridium novyi*, had the best tumour colonization ability (Dang et al., 2001). Compared with viral vectors, *Clostridia* have several advantages for cancer gene therapy, including fewer safety concerns; low immunogenicity; specific tumour-targeting; and no risk of insertional mutagenesis. Several studies have shown the tumour-specific colonization of this bacterium, and also its anti-tumour activity, either the wild-type bacterium itself or when expressing therapeutic genes (Forbes, 2010) such as the pro-drug activating gene (Liu et al., 2002). Although all of the studies demonstrated tumour inhibition, tumours were not completely eliminated. Therefore, combined therapy, for example with chemotherapeutic drugs, may achieve better results (Dang et al., 2001, Dang et al., 2004). Furthermore, the significant toxicity that was generated must be evaluated further (Dang et al., 2001).

Bifidobacteria-based Vector

Bifidobacteria is another obligate anaerobic bacterium that showed specific tumour colonization and mediated cancer gene therapy in a tumour mouse model. It was found to specifically colonize tumour sites following intravenous administration (Kimura et al., 1980). *Bifidobacteria* have several advantages over other bacteria for cancer gene therapy, as it is a common bacteria in the human intestine and may thus impose a lower safety risk, and it can be administered both orally and intravenously (Cronin et al., 2010). Several studies have reported that *Bifidobacteria*-mediated cancer gene therapy was effective (Li et al., 2003). The limitations of using *Bifidobacteria* include lower tumour colonization, non-oncolysis and no spore-formation (Wei et al., 2007). Spores are not only easy to produce, stable and economical, but also only germinate when they encounter requisite anaerobic conditions of tumours and thus mediate more specific tumour colonization (Wei et al., 2007).

Facultative Anaerobes

Facultative anaerobes, such as *Salmonella* and *Escherichia*, are also potential delivery vehicles for cancer gene therapy (Wei et al., 2007) and were thought to flood into tumours following inflammation (Leschner et al., 2009), to migrate towards tumours by chemotaxis (Kasinskas and Forbes, 2006, Kasinskas and Forbes, 2007) and to exhibit preferential growth in tumour microenvironments (Zhao et al., 2005).

Salmonella-based Vector

Initial studies using attenuated *Salmonella typhimurium* for cancer gene therapy showed excellent tumour colonisation with limited pathogenicity (Saltzman et al., 1997). The first systemic delivery study also showed suppression of tumour growth and prolonged animal survival rate (Pawelek et al., 1997), and most *Salmonella typhimurium* was found to only survive in tissues that became necrotic (Forbes et al., 2003). So far, *Salmonella*-based cancer gene therapy has been studied using different strategies in a variety of tumour models (Moreno et al., 2010), and its efficacy has been demonstrated both orally (Jia et al., 2007, Chen et al., 2009) and by intravenous administration (Ryan et al., 2009). One of the major drawbacks of *Salmonella*-mediated cancer gene therapy observed in one clinical trial is that the ratio of colonisation of tumour to normal tissue is comparatively low, due to it being a facultative anaerobe. (Nemunaitis et al., 2003).

E.Coli-based Vector

Probiotic *Escherichia coli* Nissle 1917 strain was first seen to efficiently colonize and to replicate at the necrotic border of tumour tissue in a mouse model (Stritzker et al., 2007), and has since been demonstrated to effectively colonize tumours and to mediate anticancer gene therapy alone (Forbes, 2010), and when combined with radiotherapy (Jiang et al., 2010).

Although many studies have shown the potential of anaerobic bacteria for cancer gene therapy, no reports have yet demonstrated complete tumour shrinkage (Wei et al., 2008) and challenges still remain to be addressed before bacteria can be used in clinical cancer treatment, such as the intrinsic bacterial toxicity, its tumour-targeting

efficiency and its efficiency in combination with other therapies (Forbes, 2010).

Cellular Vehicles

Despite the fact that many strategies have been developed for cancer gene therapy, physiological barriers and toxicity often limit their efficacies *in vivo*. Cellular vehicles have gained increasing interest following the observation that certain cells have the intrinsic ability to migrate to tumour sites (Roth et al., 2008).

Mesenchymal Stem Cell-based Vehicle

Mesenchymal stem cells (MSC), which can be isolated from bone marrow (Friedenstein et al., 1987), adipose tissue (Zuk et al., 2001) and placenta (In 't Anker et al., 2004), are multipotent adult stem cells that can differentiate into several mesenchymal lineage tissues, such as bone and cartilage-forming cells, under certain conditions (Friedenstein et al., 1987). It was also found that individual MSC can retain their multipotent ability when expanded *in vitro* (Pittenger et al., 1999). Since the finding that MSCs migrate to tumour sites, they have emerged as a potential cellular vehicle to target tumours, and several properties of MSC have made them attractive subjects for the delivery of therapeutic genes for cancer therapy (Hu et al., 2010). MSC can be easily isolated from a variety of tissue sources and are easy to expand *in vitro* (Compte et al., 2009). It was reported that they can be passaged more than 50 times *in vitro* without losing their multipotency phenotype (In 't Anker et al., 2004). MSC have also showed a high level of migration towards tumour sites (Studeny et al., 2002). They should present low immunogenicity associated with their lack of or low level of MHC class I and class II surface molecules, as well as the absence of co-stimulatory molecules. No immune response was observed when MSC were engrafted into the bone marrow of recipients for 13 weeks after transplantation (Saito et al., 2002) and it was shown that the viral transduction of MSC with therapeutic genes *in vitro* did not affect their stem cell properties (Chan et al., 2005). The natural tumour-homing ability of MSC should also be more efficient than other targeting strategies, such as the ligand–receptor interactions. All of these features promote MSC as a useful cellular vehicle.

Although the exact mechanism involved in MSC homing to tumour sites has not been entirely established, increasing evidence has revealed that the tumour microenvironment plays an important role in the migration of MSC, which are recruited by the chemokines (Klopp et al., 2007, Dwyer et al., 2007) and cytokines (Ries et al., 2007) released by tumour tissues (Xu and Zhu, 2007). Currently, C-X-C chemokine receptor type 4 (CXCR4) and its receptor, stromal cell-derived factor-1 (SDF-1), are considered to be the most important cytokine pair for recruiting MSC to tumours (Hu et al., 2010).

So far, MSC have been found not only to migrate towards tumour cells *in vitro* (Nakamizo et al., 2005, Schichor et al., 2006), but have also migrated specifically to tumour sites in a variety of xenograft tumour mouse models (Hu et al., 2010) including ovarian (Kidd et al., 2009), glioma (Nakamizo et al., 2005), melanoma (Studeny et al., 2002) and breast carcinoma (Studeny et al., 2004) and in an established lung metastases mouse model (Xin et al., 2007).

MSC loaded with therapeutic genes were found to be effective in mediating therapeutic transgene expression in tumour sites. After the intravenous administration of MSC transduced with therapeutic genes in human breast cancer xenograft and melanoma xenograft tumour models, tumour growth was suppressed and prolonged animal survival was achieved (Studeny et al., 2004). This has been observed in various tumour models using different therapeutic genes (Xin et al., 2007, Kucerova et al., 2007, Ren et al., 2008, Loebinger et al., 2009). MSC-based gene therapy also faces several challenges. It was reported that *in vitro* expanded MSC showed less tumour-homing ability (Potapova et al., 2008). And there is growing evidence that human MSC are involved in cancer initiation as a consequence of their ability to spontaneous transform *in vitro* (Rubio et al., 2008) with higher frequency than other cell types and to contribute to tumour growth *in vivo* (Li et al., 2007a). Thus, a greater understanding of the true fate of MSC *in vivo* is needed before they can be used as cellular vehicles for cancer gene therapy (Hu et al., 2010).

In addition, neural stem cells have also emerged as potential cellular vehicles for brain tumour gene therapy, as they were found to engraft in brain tumour sites after systemic delivery (Ahmed et al., 2010).

Endothelial Lineage Cell-based Vehicle

In 1997, the discovery of CD34-enriched endothelial progenitor cells (EPC) (Asahara et al., 1997) changed our belief that the formation of new blood vessels occurred exclusively via the mechanism of sprouting and intussusceptive angiogenesis (Folkman and Shing, 1992). EPC are defined as precursor cells that can differentiate into mature endothelial cells when attracted to angiogenic sites and are believed to be differentiated from haemangioblasts, the haematopoietic stem cells (Rafii and Lyden, 2003). EPC can be isolated from both bone marrow and peripheral blood (Ferrari et al., 2003) and were recently also observed in vascular walls (Ingram et al., 2005, Zengin et al., 2006). Over the past few years, a substantial number of reports from animal and human studies have contributed to our understanding of EPC, and we now know that they are involved in tumour vasculogenesis and are critical to tumour neovascularisation; can migrate towards angiogenic signals; and that they can proliferate to form tubular structures required for the formation of all blood vessels and capillaries (Dome et al., 2007). Inflammatory cytokines were also reported to contribute to engraft EPC to tumours (Spring et al., 2005).

Increased numbers of circulating EPC have been found in many human cancer patients and levels of circulating EPC correlate with progression of tumours such as multiple myeloma (Zhang et al., 2005a; Rigolin et al., 2006), acute myeloid leukaemia (Wierzbowska et al., 2005, 2008; Rigolin et al., 2007), chronic lymphocytic leukaemia (Rigolin et al., 2010), hepatocellular carcinoma (Ho et al., 2006), non-small cell lung cancer (Dome et al., 2006), lung cancer (Nowak et al., 2010), lymphoma (Igreja et al., 2007), glioma (Zheng et al., 2007), classic Kaposi's sarcoma (Taddeo et al., 2008), head and neck cancers (Brunner et al., 2008), ovarian cancer (Su et al., 2010) and pancreatic carcinoma (Vizio et al., 2010). Increased circulating EPCs have also been observed after chemotherapy (Bertolini et al., 2003, Furstenberger et al., 2006, Shaked et al., 2006).

The incorporation of EPC in tumours was discovered for the first time when β-galactosidase labelled-EPCs were found both in tumour stroma and tumour endothelium (Asahara et al., 1999). Since then, many groups have reported the homing ability of EPC in different tumour model systems. Using a mathematical model, EPCs were predicted as having a significant role in the early stages of tumour growth (Stoll et al., 2003, Suriano et al., 2008). Bone-marrow-derived EPC were found to incorporate into tumour sites in spontaneous mammary tumour (Nolan et al., 2007) and malignant xenograft glioma models (Zhang et al., 2009). Blocking the recruitment of EPC (Murakami et al., 2009) or inhibiting the expression of DNA binding fator-1 in EPC inhibited tumour growth (Gao et al., 2008, Mellick et al., 2010), and these data indicated the contribution of EPC to tumour neovascularisation. EPC are not only found migrating to tumour sites, but are also observed to contribute to neovascularization under other conditions, such as during wound healing (Asahara et al., 1999), induced hindlimb ischaemia (Takahashi et al., 1999, Kalka et al., 2000), experimental myocardial infarction (Jackson et al., 2001), venous thrombosis (Modarai et al., 2005), endothelial denudation (Ii et al., 2006, Urao et al., 2006) and diseased kidney containing microvascular injury (Hohenstein et al., 2010). The specific tumour-homing ability of EPC has made them attractive cellular vehicles for cancer gene therapy. After intravenous administration, bone marrow-derived EPC modified to express thymidine kinase genes have been reported to migrate to tumour sites, resulting in extensive tumour necrosis occurring in a xenograft glioblastoma mouse model (Ferrari et al., 2003) and in a glioma xenograft tumour model (Zhang et al., 2010).

Blood late outgrowth endothelial cells (BOEC) are derived from a subset of CD34-enriched peripheral blood cells that can differentiate into endothelial cells (Shi et al., 1998). BOEC have been observed to migrate to Lewis lung carcinomas and lung metastases after being injected intravenously (Wei et al., 2007a), and BOEC genetically modified to express the angiogenic inhibitor, endostatin, decreased tumour vascularisation and tumour volume (Dudek et al., 2007).

The intravenous injection of other endothelial lineage cells, such as embryonic EPC, can target hypoxic lung metastasis and prolong animal survival when harbouring an exogenous suicide gene, yeast cytosine deaminase (CD) gene fused to a uracil phosphoribosyl transferase (UPRT) (Wei et al., 2004). Mature murine lung endothelial cells expressing interleukin-2, when injected systemically, can migrate to lung metastases, decrease tumour burden and prolong animal survival (Ojeifo et al., 2001).

3.2.2 RNAi-based Technology

3.2.2.1 The Discovery of RNAi

Although ssRNA was initially shown to silence specific genes as a result of binding to the target mRNA (Fire et al., 1991), the exact mechanism was not understood until RNA interference (RNAi) was discovered. RNAi is a natural phenomenon that was first found in the nematode worm *Caenorhabditis elegans*, in which the presence or introduction of long double-stranded RNA (dsRNA) molecules can degrade sequence-specific mRNA molecules, and the dsRNA-mediated mRNA degradation is more effective than either strand alone (Fire et al., 1998). The two scientists Andrew Z. Fire and Craig C. Mello, who discovered this natural phenomenon, were awarded The Nobel Prize in Physiology or Medicine in 2006. RNAi is now recognized as a highly conserved biological process that occurs in cells of almost every multicellular organisms, and may have evolved as an essential posttranscriptional gene regulation system and/or as a defence mechanism against viral dsRNA (Hannon, 2002). It was later reported that RNAi was mediated by dsRNA molecules 21–22 nucleotides in length in the *Drosophila* system (Elbashir et al., 2001b) and in cultured mammalian cells (Wianny and Zernicka-Goetz, 2000, Elbashir et al., 2001a).

3.2.2.2 Mechanism of RNAi

The mechanism of RNAi begins with the processing of long dsRNA molecules (Tuschl et al., 1999). These dsRNAs which may either be expressed intracellularly or introduced into the cells from an exogenous source are processed into 21–23bp siRNA fragments (Zamore et al., 2000) by the action of an RNAse III called Dicer

(Bernstein et al., 2001). This Dicer enzyme specifically recognizes and binds to the 5' phosphate of dsRNA precursor and makes two staggered cuts in both strands of the dsRNA to causes 2-nucleotide 3' overhangs (Elbashir et al., 2001b). This siRNA is produced by Dicer complexed with the dsRNA-binding protein (TRBP/TARBP2P) and the protein kinase R (PKR)-activating protein (PACT) (Kok et al., 2007). The complex of siRNA, Dicer and TRBP–PACT is then loaded onto the RNA-induced silencing complex (RISC) (Hammond et al., 2000). This requires the action of the RISC loading complex (RLC) that consists of the double-stranded siRNA, Dicer and the dsRNA-binding-domain protein, R2D2 (Liu et al., 2003). R2D2 also helps to determine the asymmetry of the siRNA by binding to the more thermodynamically stable end of the siRNA (Tomari et al., 2004). The RLC initiates siRNA unwinding and determines which strand is to be assembled into the RISC by preferentially binding the strand with the highest thermodynamic stability at the 5′ end (Schwarz et al., 2003), and this becomes the guide strand (Martinez et al., 2002) and usually the antisense strand (Zamore et al., 2000). The other strand, named the passenger strand is cleaved by Ago2, an endonuclease and a core component of RISC (Matranga et al., 2005), and leaves the complex, thereby facilitating activation of RISC (Rand et al., 2005). The phosphorylated 5′-terminal nucleotide of the guide strand inserts into a positively charged pocket on Ago2 and consequently does not participate in the recognition of and binding to the target mRNA (Ma et al., 2005). Nucleotides 2–8 of the guide strand are exposed and directly recognize the target mRNA (Ma et al., 2005, Lewis et al., 2005). The mRNA is then cleaved by Ago2 (Liu et al., 2004a) at the centre of the oligonucleotide duplexed with the guide strand (Martinez and Tuschl, 2004). The RISC remains bound to the siRNA and can execute subsequent rounds of cleavage of target mRNA molecules (Hutvagner and Zamore, 2002) (Figure 7). Unlike plants and worms, which can replicate siRNA, there is no evidence of siRNA replication in mammals (Zamore, 2002). Thus, siRNA-mediated gene silencing in mammalian cells is limited by its transient nature.

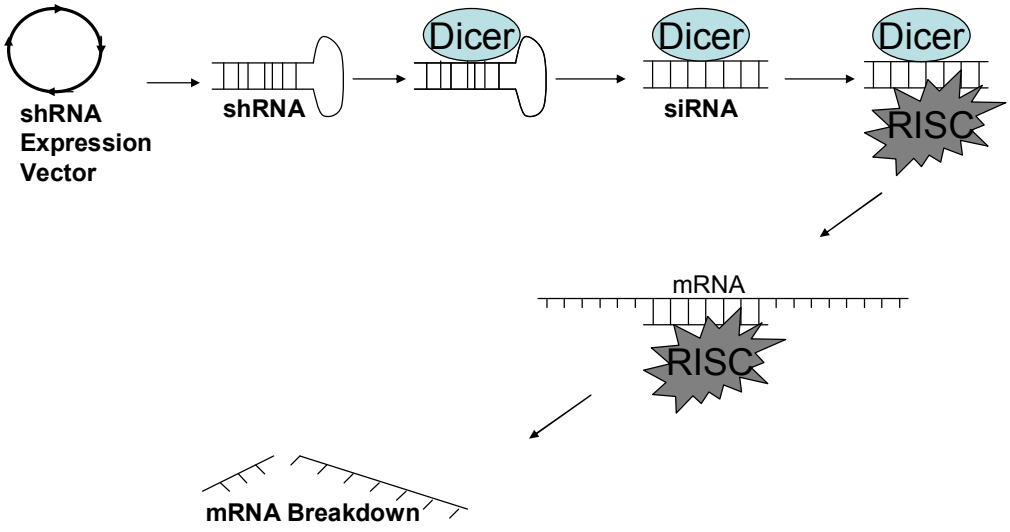


Figure 7 – The Mechanism of RNAi - Schematic

In mammalian cells, RNAi can be triggered either by DNA-based shRNA expression vector (as used in this project) or by direct small siRNA molecules. In each case, gene silencing is caused by the destruction of mRNA that is complementary to the input siRNA.

3.2.2.3 Vector-based RNAi Induction

It has been demonstrated that siRNA can be expressed intracellularly from a DNA plasmid vector encoding a short hairpin RNA (shRNA) (Brummelkamp et al., 2002b) (Paddison et al., 2002). The shRNA transcript has a hairpin-like stem-loop structure which is processed in the nucleus by the microprocessor complex (Lee et al., 2003). After being exported to the cytoplasm by exportin-5 (Yi et al., 2003), the hairpin loop is removed by the Dicer–TRBP–PACT to form the siRNA (Zeng et al., 2003). Plasmid-based shRNA must be chemically transfected like siRNA, and may thus exhibit low transfection efficiency on primary cells and difficulty in integrating into the host genome for stable expression (Masiero et al., 2007).

To overcome these problems, siRNA viral vectors have been designed via the expression of shRNA to achieve more efficient and extended gene silencing effects, including in some primary cells (Brummelkamp et al., 2002a, Stewart et al., 2003, Rubinson et al., 2003).

3.2.2.4 siRNA-based Telomerase Inhibition

There have been many reports regarding siRNA-based approaches targeting both of the core components of human telomerase, hTERT and hTR. The first report of utilising siRNA to inhibit TERT and TR showed reduced telomerase activity in a variety of human cancer cell lines, including both carcinomas and sarcomas, and the results showed that targeting TR was more effective than targeting TERT (Kosciolek et al., 2003).

Many groups have utilised different forms of siRNA to inhibit TERT in a variety of human cancer cell lines for shorter or longer periods, and they have reported significant inhibition of telomerase activity along with either decreased cell proliferation or increased apoptosis (Kurvinen et al., 2006, Miri-Moghaddam et al., 2009, Wang et al., 2007, Zhang et al., 2010b). Targeting TERT by siRNA *in vivo* in different tumour models also caused inhibition of tumorigenicity, tumour growth and the invasive capacity of tumours (Pallini et al., 2006, Gandellini et al., 2007).

A recent report comparing siRNA-mediated inhibition of either hTR or hTERT in human oral cancer cells showed that targeting hTR caused the greatest telomerase inhibition result *in vitro* and the largest reduction in tumour growth *in vivo* (Li et al., 2011). It has been further demonstrated that targeting TR using naked siRNA (Zheng et al., 2006), plasmid shRNA vectors or viral shRNA vectors caused significant telomerase inhibition and either reduced cell proliferation or increased apoptosis in various human cancer cells, including lung cancer cells (Li et al., 2004b) and renal cancer cells (Zheng et al., 2006). Furthermore, additive effects on inhibition of cell proliferation were observed with siRNA combined with other anticancer treatments in various human cancer cell lines (Goldkorn and Blackburn, 2006) and mouse tumour models (Xu et al., 2010). Other *in vivo* reports also showed that siRNA targeting telomerase components inhibited tumour growth in tumour mouse models (Li et al., 2007b).

3.2.3 Aims of this Chapter

Having already developed an effective siRNA viral system for telomerase inhibition

in vitro, I aimed to explore novel delivery strategies, including cellular vehicles and synthetic dendrimers, for the efficient delivery of the siRNA vector *in vitro* and *in vivo*. In addition, I aimed to develop a DNA plasmid system for siRNA targeting canine telomerase. Specifically I aimed to answer the following questions in this chapter:

1. What is the transfection efficiency of endothelial cells *in vitro*?
    - A protocol for the transient transfection of a β-galactosidase vector using Lipofectamine™ 2000 was first established in 293FT cells.
    - A series of transfection experiments was conducted in three endothelial cell lines, EOMA, HUVEC and SB-HSA cells, with a β-galactosidase reporter vector using four different commercial transfection reagents: Effectene, Fugene 6, Tfx-50 and Lipofectamine™ 2000.
    - The transient transfection of SB-HSA cells with a GFP reporter vector were further optimised using a modified Lipofectamine™ 2000 protocol.
    - SB-HSA cells were stably transfected with a luciferase vector using Lipofectamine™ 2000.

2. Can endothelial cells migrate to the tumour formation site *in vivo*?
    - Endothelial SB-HSA cells were investigated as a potential cellular vehicle: they were labelled then intravenously administered and tissue distribution was analysed in a canine xenograft tumour mouse model.

3. What is the transfection efficiency of PPIG3 dendrimers in cells?
    - The transfection efficiency of SB-HSA cells and D-17 cells were explored using novel PPIG3 dendrimers complexed with a GFP vector.

4. Can siRNA plasmid expression vector silence the canine telomerase RNA component *in vitro*?
    - Following optimization of the transfection efficiency of SB-HSA cells,

the transfection efficiency of another canine cell line, D-17, was also tested.

- A plasmid siRNA expression vector targeting cTR was designed, constructed and tested on canine SB-HSA cells and D-17 cells.

3.3 Materials and Methods

3.3.1 Transient Transfection of 293FT Cells and All Endothelial Cells

293FT, EOMA, HUVEC and SB-HSA cells were transiently transfected with pSV-β-galactosidase control vector (Promega, United Kingdom) using four different commercial chemical reagents: Lipofectamine™ 2000 (Invitrogen, United Kingdom), Tfx™-50 Reagent (Promega, United Kingdom), Fugene 6 (Roche, United Kingdom) and Effectene (Qiagen, United Kingdom). All transfections were performed in 24-well plates (Corning, United Kingdom) in triplicates except for 293FT cells (Corning, United Kingdom) where 6-well plates were used; each well was used for a single transfection reaction, and mock transfection and untreated cell controls were included. When analyzing transfected cells, after examining the whole plate, three microscopic fields of transfected cells which represented the best of the whole plate were photographed and one photo was chosen and presented in the result section.

3.3.1.1 Lipofectamine 2000 Standard Protocol

24h before transfection, sufficient cells were plated so that cells would be approximately 90% confluent at the time of transfection. For each transfection, 2µl of Lipofectamine™ 2000 was first diluted with 50µl Opti-MEM® I Reduced Serum Medium (Invitrogen, United Kingdom) and incubated at room temperature for 5–25 min. 0.8µg DNA was then diluted with 50µl Opti-MEM® I Reduced Serum Medium. The solutions were then combined, gently mixed then incubated for 20 min at room temperature. The mixture was then was added to the cell medium. After transfection, cells were grown in a 37˚C incubator, monitored regularly and analysed after 48h.

3.3.1.2 Tfx™-50

24h before transfection, sufficient cells were plated so that cells would be approximately 80% confluent at the time of transfection. Prior to transfection, a stock solution of Tfx™-50 reagent was prepared by resuspending one vial of reagent in 400µl NF-water and vortexed, followed by incubation in a 65ºC water bath for 1min. Before use, the Tfx reagent stock solution was thawed at room temperature and thoroughly mixed. 1µg of DNA was first mixed with 3µl Tfx reagent, then diluted with growth medium to a final volume of 200µl and incubated at room temperature for 15 min. After incubation, the medium was removed from the cells and the reagent mixture was added. After 1h incubation at 37ºC, another 1ml growth medium was gently overlaid onto the cells and the plate was return to the incubator for 48h until analysis.

3.3.1.3 Fugene 6

24h before transfection, sufficient cells were plated so that cells would be approximately 80% confluent at the time of transfection. 1.5µl reagent was first diluted with serum-free medium then 0.25µg DNA in 23.5µl of serum-free medium was added to the diluted reagent to make a final volume of 25µl. The mixture was vortexed for 1s then added to the cells in a drop-wise manner. Cells were then put back to the incubator until 48h later for analysis

3.3.1.4 Effectene

24h before transfection, sufficient cells were plated so that cells would be approximately 80% confluent at the time of transfection. For transfection, 0.2µg DNA was diluted in 60µl EC buffer followed by the addition of 1.6µl enhancer and vortexing for 1s. This mixture was incubated at room temperature for 5min, then 5µl Effectene reagent was added to the enhancer mix and mixed by vortexing for 10s. The mixture was then incubated at room temperature for 10min to allow complex formation to occur. The cell medium was aspirated from the cells, which were then washed with PBS, followed by addition of 350µl fresh medium to each well. The transfection mix was then diluted with 350µl medium and then added to the cells in a

drop-wise manner. Cells were incubated at 37ºC for 48h before analysis.

3.3.2 *In Situ* Staining of Cells for β-galactosidase Activity

Directly before staining, glutaraldehyde solution and x-gal solution were freshly prepared. 0.25% glutaraldehyde solution was prepared by mixing 25µl 100% glutaraldehyde solution (Sigma, United Kingdom) with 9975µl PBS. 0.2% x-gal solution was prepared by 2% x-gal (Sigma, United Kingdom), 2mM $MgCl_2$ (Sigma, United Kingdom), 5mM $K_4Fe(CN)_6.3H_2O$ (Sigma, United kingdom) and $K_3Fe(CN)_6$ (Sigma, United Kingdom) in 10ml PBS.

48h after transfection, cell medium was aspirated and cells were washed 3 times in PBS. 0.2ml glutaraldehyde solution was added to each well and incubated for 15min. The glutaraldehyde solution was then removed and cells were then washed 3 times with PBS to remove residual glutaraldehyde that could inhibit β-galactosidase activity. 0.1ml x-gal solution was then added to each well and cells were incubated at 37˚C until they were visibly stained. X-gal was then removed and replaced with PBS for microscopic analysis.

3.3.3 Lipofectamine 2000 Modified Protocol

After replacing β-galactosidase vector with a GFP plasmid vector, pmaxGFP (Lonza, United Kingdom), instead of plating cells 24 hours before transfection, cells were plated on the day of transfection to reach approximately 90% confluency followed by adding DNA-transfection reagent complex. This protocol was used on SB-HSA cells and D-17 cells to compare with the standard protocol and used in all experiments from then.

3.3.4 Stable Transfection

SB-HSA cells were stably transfected with a pGL4.18 luciferase expression vector containing a neomycin resistant gene (Promega, United Kingdom) using Lipofectamine 2000 modified protocol. Before transfection, G418 was titrated into SB-HSA cells to determine their drug sensitivity and the optimal plating density was

then determined.

3.3.4.1 G418 Titration

2 x 10^4 SB-HSA cells were plated into each well of a 24-well plate containing 1ml of culture medium. After 24h, 500µl culture medium containing either 0, 25, 100, 300, 500, 1000 or 4000µg/ml G418 (Invitrogen, United Kingdom) was added to triplicate wells. Cells were cultured for 10–14 days and the correct concentration of G418-containing medium was replaced every three days. Cells were examined for viability and photographed every 2 days. This experiment was designed to identify the lowest G418 concentration that induced cell death in approximately 7–9 days and that killed all cells within 2 weeks. This concentration was then used to select cells transfected with the pGL4.18 luciferase expression neo vector.

3.3.4.2 Optimization of Plating Density

Different amounts SB-HSA cells (0.25 x 10^5, 0.5 x 10^5, 1 x 10^5 and 2 x 10^5) were plated in triplicate wells of a 24-well plate containing 1ml of culture medium in triplicates. After 24h, 500µl culture medium containing G418 was added using 250µg/ml, the concentration determined earlier in the G418 titration experiment. Cells were then cultured for 5–14 days and G418-containing medium was renewed every 3 days. In this way, a cell plating density was identified that allowed the cells to reach 80% confluency before cell death occured, and this density was used to plate SB-HSA cells transfected with the pGL4.18 luciferase expression neo vector.

3.3.4.3 Stable Transfection of SB-HSA Cells

SB-HSA cells were transfected with pGL4.18 luciferase expression vector (Promega, United Kingdom) containing a neomycin resistance gene using Lipofectamine 2000. The modified transfection protocol mentioned earlier was followed (3.3.3).

After transfection, cells were incubated for 48h, then G418-containing medium was added and transformed cells were selected for one week using the G418 concentration and cell seeding density determined previously. During this time cells

were monitored regularly. After a week cells were harvested for the luciferase assay to examine if stable-transfection was successful. In order to measure the long-term gene expression of luciferase, stably-transfected cells were continuingly grown in two separate flasks: one flask of cells was grown in normal growth medium and the other flask was grown in medium containing 125µg/ml G418. After 6 months, cells in both flasks were assayed for luciferase activity.

3.3.4.4 Luciferase Assay

The luciferase assay was performed on stably transfancted cells using the Bright-Glo™ Luciferase Assay System (Promega, United Kingdom) to measure the luciferase expression. All reagents and cells were equilibrated to room temperature starting the assay. First, 1 x 10^3 luciferase-transfected or untransfected SB-HSA cells were plated one day before starting the assay in 100µl culture medium in a 96-well plate (Corning, United Kingdom). After 24h, the culture medium was removed completely and cells were carefully washed 3 times with PBS. 100µl Glo Lysis Buffer (Promega, United Kingdom) was then added and plates were gently rocked to ensure an even coverage. Cells were then incubated at room temperature for 5min for cell lysis. After the incubation, the cell lysate was transferred to a 96-well luminometer plate, followed by the addition of 100µl Bright-Glo™ Luciferase Assay Reagent. The luminescence from each cell lysate was then measured immediately 3 times within the first two min using a Wallac 1420 Multilabel Counter, and corrected against empty well controls. The values of luminescence from each group of stably-transfected cells, untransfected cells and empty wells were used to generate the column.

3.3.4.5 Irradiation of SB-HSA-Luc Cells

For γ irradiation, triplicate samples of 5 x 10^5 SB-HSA-Luc cells were subjected to doses of 5Gy, 10Gy or 20Gy. The original dose from the γ-irradiator was 16.02Gy/min, and the conversion factor 0.8606 was used to determine the radiation emitted in June 2009. The irradiation time for the required doses was then calculated as follows:

Exposure time = required dose x conversion factor / original dose

After irradiation, cells were then cultured for a week before the luciferase assay was done.

3.3.5 *Ex Vivo* Imaging

3.3.5.1 Mouse strain

10 NOD-SCID female mice (Charles River, United Kingdom) were housed in three groups in the animal unit at 19–23˚C with a 12h light–dark cycle, and fed with a conventional diet with mains water *ad libitum* at Little France Small Animal Unit, University of Edinburgh. All mice experiments were conducted with appropriate ethical approval and in accordance with the U.K. Home Office regulations.

3.3.5.2 Tumour Initiation

10 mice were each injected subcutaneously with 3 x 10^7 SB-HSA cells in their right flanks and tumour growth was monitored regularly until they reached the length of approximately 10mm.

3.3.5.3 Cell-labelling

5µl DiR dye (50mg/ml stock solution in dimethylsulfoxide (DMSO), from Dr Kev Dhaliwal) was added to 5 ml 1 x 10^8 SB-HSA cells in suspension. After 5min room temperature incubation, cells were pelleted and supernatant was removed. Cells were then resuspended by PBS and centrifuged again. This PBS washing process was then repeated twice to discard any free dye left in the solution. At the last time of washing, cells were resuspended in growth medium. Labelled SB-HSA cells were then kept on ice in the dark and used immediately for mouse injections.

3.3.5.4 *Ex Vivo* Cell Trafficking

Before injection, all injected solutions were warmed in a 37˚C water bath then 1 x 10^7 labelled SB-HSA cells in 200µl medium were administered intravenously into the tail vein of 4 mice. As a control, 1µl DiR stock was diluted 1:1000 1ml growth

medium, corresponding to the same dilution of the cell-staining process. 200µl diluted dye was then administered intravenously into 3 mice. All mice, including 3 untreated control mice, were euthanized 24h after injection by a standard procedure and all major organs were collected, including the liver, spleen, kidney, heart and hind limbs. *Ex vivo* fluorescent imaging was then performed using a Xenogen IVIS 200 imaging system combined with Living Image (Version 3.2.0.8156) software.

3.3.6 Optimization of Transfection Efficiency Using PPIG3 Dendrimers

3.3.6.1 Extra Desalting of Plasmid DNA

The GFP plasmids used in this experiment were purified with an extra desalting procedure using Illustra NAP-10 Columns (GE Healthcare, UK) to assist effective nanoparticle formation. Excess NF-water was firstly passed through the column for rehydration, then another 15 ml NF-water was passed through for equilibration. A 1ml sample of plasmid DNA was then added to column, and desalted plasmid DNA was eluted using 1.5ml NF-water.

3.3.6.2 Preparation of Nanoparticles

After desalting, GFP plasmids were sent to Dr Andreas Schatzlein's laboratory (University of London) by special courier for nanoparticle assembly. The ratio of DNA to PPIG3 dendrimers was kept at a constant 1:5 in 5% dextrose solution and nanoparticle formation was checked before being posted back to our laboratory.

3.3.6.3 Transfection Protocol Using PPIG3 Dendrimers on Canine Cells

A transfection efficiency tests was performed on both SB-HSA cells and D-17 cells using these dendrimers and is independent from those transfection test performed earlier using commercial transfection reagents (3.3.1). This was done in 24-well plates with the GFP plasmid complexed to PPIG3 dendrimers, and 1.5µg, 3µg, 4.5µg and 6µg DNA in triplicate were compared for optimizing transfection conditions. For transfection, Opti-MEM® I Reduced Serum Medium was used to dilute the

nanoparticles to a final volume of 250 µl. Cells were seeded to reach an approximate 60% confluency and diluted nanoparticles were added to each well. Experimental controls were PPIG3 dendrimers only in 5% dextrose as mock transfections and untreated cell controls. After transfection, cells were grown in a 37ºC CO_2 incubator for 96h. Cells were checked regularly and photographed at 48h and 96h after transfection using a fluorescent microscope fitted with a black and white camera.

3.3.7 Preparation of siRNA

3.3.7.1 Construction of siRNA Plasmids

The plasmid-based siRNA vector was constructed using the psilencer 4.1-CMV neo siRNA expression vectors kit, which also contains a siRNA insert targeting human GAPDH gene and ready-to-use scrambled siRNA to be used as negative controls. Construction of all siRNA expression vectors was done following the manufacture's protocol. All components mentioned below were provided by the kit unless otherwise stated. Oligonucleotides of cTR or hGAPDH inserts were first dissolved in 100µl NF-water each, then diluted in TE buffer to obtain an approximate concentration of 1µg/µl. 50µl of annealing mixture was assembled from: 46µl 1X DNA annealing solution; 2µl diluted sense oligonucleotides; and 2µl diluted antisense oligonucleotides. The annealing mixture was heated in a heating block to 90˚C for 3 min, and then cooled in a 37˚C incubator for 1h. 5µl of the annealed product was then diluted with 45µl NF-water to obtain a concentration of 8ng/µl. Two sets of 10µl ligation reactions were then set up: a plus-insert ligation and the minus-insert negative control. For ligation reactions (plus-insert), 1µl diluted annealed siRNA insert, 1µl 10X T4 DNA ligase buffer, 1µl psilencer 4.1-CMV neo vector, 1µl T4 DNA ligase and 6µl NF-water were combined. In control reactions (minus-insert), 1µl diluted annealed siRNA insert was replaced by 1µl 1X DNA annealing solution. All ligation reactions were incubated at room temperature for 3h. Ligation products from both sets of reactions were transformed into competent cells, which were then plated and incubated overnight at 37°C. The number of colonies on all plates (plus-insert, minus-insert or non-transformed competent cells) were then counted. Ideally, the plate containing non-transformed cells should yield no colonies, and the plate

containing cells transformed with the plus-insert reaction products should yield 2–10-fold more colonies than the plate containing cells transformed with the minus-insert reaction products. Several clones were picked from each plate and plasmids were isolated using a mini kit (2.2.8.1) to screen for positive ligation products.

3.3.7.2 Confirmation of shRNA Insert

Restriction Enzyme Digestion

Isolated siRNA plasmid DNA was subjected to BamHI and HindIII (Promega, United Kingdom) double restriction enzyme digestion to confirm the presence of the 55bp cTR or hGAPDH shRNA insert. The reaction recipe for restriction enzyme digestion was shown below (Table 5). After mixing all components gently, it was incubated at 37°C for 4h.

Component	Amount (µl)
Restriction Enzyme 10X Buffer E	2
Acetylated Bovine Serum Albumin, 10µg/µl	0.2
Constructed siRNA Vector	1µg
BamHI, 10U/µl	0.5µl
HindIII, 10U/µl	0.5µl
NF-water	To a final volume of 20µl

Table 5 – Double Restriction Enzyme Digestion

All components were provided in the kit, unless otherwise stated. Buffer E and 37°C incubation are the optimal reaction buffer and temperature for both restriction enzymes.

DNA Extraction from Agarose Gel

After double-restriction enzyme digestion, the product was run on a 2% agarose gel. Gels were then photographed under UV light and correspondent bands were excised. Excised GAPDH and cTR band on agarose gel were recovered using Wizard® SV Gel and PCR Clean-up system. While visualizing the DNA, correspondent DNA band was excised from the gel quickly to reduce the UV-damage to the DNA and transferred into a microcentrifuge tube. The gel slice was then weighed and 10µl Membrane Binding Solution per 10mg agarose gel was added into the tube. The mixture was vortexed and incubated at 65°C until the gel was completely dissolved.

The dissolved gel slice mixture was then transferred to the SV minicolumn assembly and incubated at room temperature for 1 min. After incubation, the assembly was centrifuged at 16,000 x g for 1 min. After discarding the liquid, 700µl Membrane Wash Solution previously diluted with 95% ethanol was added into the assembly and centrifuged at 16,000 x g for 1 min. After emptying the assembly, the wash was repeated with 500µl Membrane Wash Solution at 16,000 x g for 5 mins. The flowthrough was then discarded and the assembly was centrifuged at 16,000 x g for 1 min with the lid open to allow evaporation of any residual ethanol. After centrifugation, the column was transferred to a new tube and 50µl NF-water was added in. It was then incubated at room temperature for 1 min followed by centrifugation of 16,000 x g for 1 min. The eluted DNA was stored in - 20°C and sent for sequencing confirmation later.

Sequencing Confirmation

After the confirmation by restriction digest, positive plasmids were sent for sequencing. Sequencing primers for the siRNA insert in plasmid vector is shown below (Table 6). After sequencing confirmation, the positive colonies were expanded again and plasmids were isolated using Hispeed Plasmid Maxi kit (2.2.8.2).

Sequencing Primers for siRNA Insert Confirmation	
Forward Primer	Reverse Primer
AGGCGATTAAGTTGGGTA	CGGTAGGCGTGTACGGTG

Table 6 – Sequencing Primers for siRNA Insert Confirmation

This set of sequencing primers was designed by Ambion to fit the siRNA plasmid vector.

3.3.8 Duplex RT-PCR for siRNA Experiment

The GoTaq® PCR Core system kit was used for all conventional PCR reactions. All PCR reactions were done using a 50µl total volume in triplicate with two control groups. RNA control groups use RNA instead of cDNA in the reaction to detect any genomic contamination and the added RNA amount is equivalent to the amount that was used to generate the added cDNA in one reaction. The water control group used NF-water to replace the same amount of cDNA in the reaction to detect any other

contamination. The PCR mixture was subjected to an initial denaturation step of 95°C for 5min, followed by 30 cycles of denaturation at 95°C for 1min, annealing at a primer-dependant temperature for 1min and extension at 72°C for 1min, followed by a final extension step at 72°C for 10min. After completing the reactions, samples were incubated at 4°C until ready to analyse. The amounts of dNTP, MgCl2, 5X GoTaq® Taxi Buffer and GoTaq® DNA Polymerase were kept constant.

A duplex RT-PCR system (Table 7) was established for analysing the silencing effect on cTR and cGAPDH after transfection and was first demonstrated on D-17 cells and SB-HSA cells. The silencing ability of the siRNA vector was demonstrated in the human H1299 cell line using the siRNA vector against hGAPDH and siRNA expression was confirmed by singleplex PCR. Finally, siRNA plasmids were tested in the SB-HSA and D-17 cell lines. All primers used in the RT-PCR are shown below (Table 8).

Component	**Amount (µl)**
cDNA / RNA / Water	2 / RNA / Water
dNTP, 10mM	1
MgCl2, 25mM	4
5X Go Taxi Buffer	10
Human or (Canine) GAPDH Forward Primer, 100pmol/µl	0.1
Human or (Canine) GAPDH Reverse Primer, 100pmol/µl	0.1
(cTR Forward Primer, 100pmol/µl)	0.1
(cTR Reverse Primer, 100pmol/µl)	0.1
GoTaq® DNA Polymerase, 5u/µl	0.25
NF-water	Add to make 50µl total volume

Table 7 – Duplex RT-PCR Reaction for siRNA Experiment

All components mentioned above were provided by the kit unless otherwise stated.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Primer-dependant Temperature (˚C)
hGAPDH	ATCACTGCCA CCCAGAAGAC	AGGCCATGTA GACCATGAGG	54
cTR	GGCCTCCGT CTAACCCTAAC	CCAGCGGCT GACATTTTT	
cGAPDH	ATCACTGCCA CCCAGAAGAC	AGGCCATGTA GACCATGAGG	54

Table 8 – All Primer Sets Used in the siRNA Experiments

cTR and cGAPDH primers were used for duplex RT-PCR to analyze the gene-silencing effect of siRNA in canine cells. hGAPDH primers were used for singleplex RT-PCR to analyze the gene-silencing effect of siRNA in human cells.

3.4 Results

3.4.1 Transfection of 293FT Cells

As a standard producer cell line for our previously-established siRNA lentiviral vector, the transfection of 293FT cells was demonstrated first *in vitro* with a β-galactosidase vector using Lipofectamine™ 2000. Strong positive β-galactosidase staining was found in transfected 293FT cells compared with mock-transfected negative control (Figure 8).

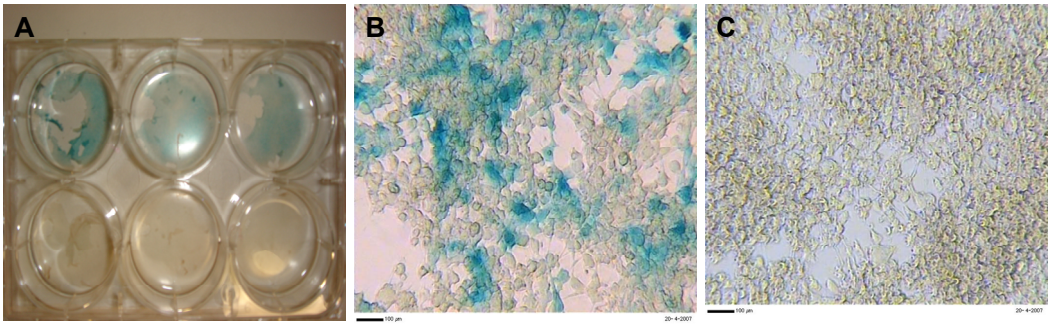


Figure 8 – Transient Transfection of 293FT Cells

A, gross appearance of transfected 293FT cells on a 6-well plate- blue colour on the plate.

B, transfected 293FT cells – blue stained

C, negative control – mock transfection of 293FT cells

3.4.2 Transfection of EOMA, HUVEC and SB-HSA Cells

As potential cellular delivery vehicles, the transfection efficiency of four different commercial transfection reagents, Effectene, Fugene 6, Tfx-50 and Lipofectamine™ 2000, were tested on three types of endothelial cell, EOMA cells, HUVEC cells and SB-HSA cells. As I expected, very low transfection efficiencies were observed generally for all three cell lines. In particular, no positive β-galactosidase staining was found in HUVEC cells using all four transfection reagents. When using Effectene, very limited β-galactosidase staining was detected in EOMA cells only (Figure 9A). However in SB-HSA cells, much more β-galactosidase staining were observed, but this was present only in when Lipofectamine 2000 was used as a transfection reagent (Figure 9B). Qualitative summary of transfection results is shown below (Table 9)

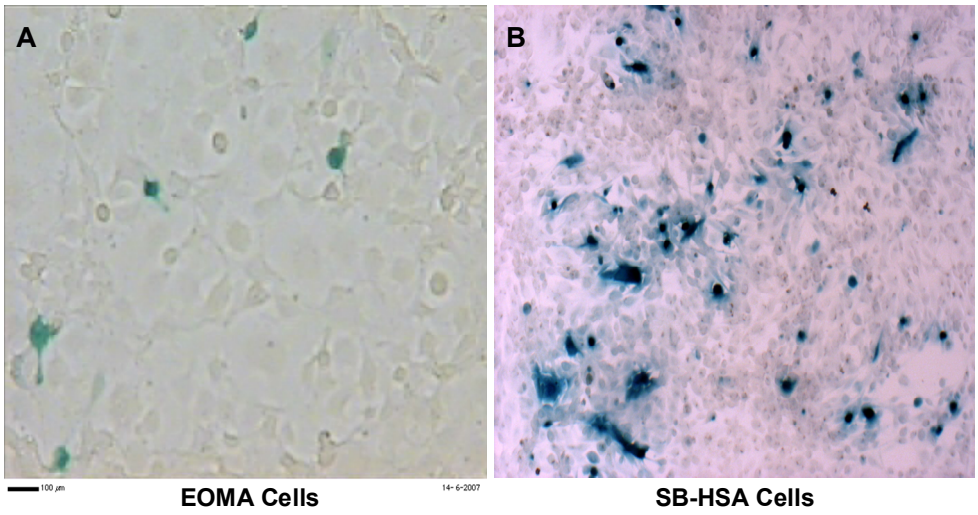


Figure 9 – Transient Transfection of EOMA and SB-HSA cells

A, EOMA cells were transfected using Effectene

B, SB-HSA cells were transfected using Lipofectamine 2000

	SB-HSA Cells	EOMA Cells	HUVEC	293FT Cells
Lipofectamine 2000	+	+	-	+
Effectene	-	-	-	NA
Tfx-50	-	-	-	NA
Fugene 6	-	-	-	NA

Table 9 – Cells with Successful Transfection by Various Reagents

Cell type which was transfected successfully was marked as + and - if not

NA – not applicable.

3.4.3 Transfection of SB-HSA Cells by the Modified Protocol of Lipofectamine 2000

The transfection efficiency of SB-HSA cells with a GFP vector using standard Lipofectamine 2000 protocol (3.3.1.1) recommended by the manufacturer was compared with using the modified protocol. The transfection efficiency was strongly increased as seen below (Figure 10)

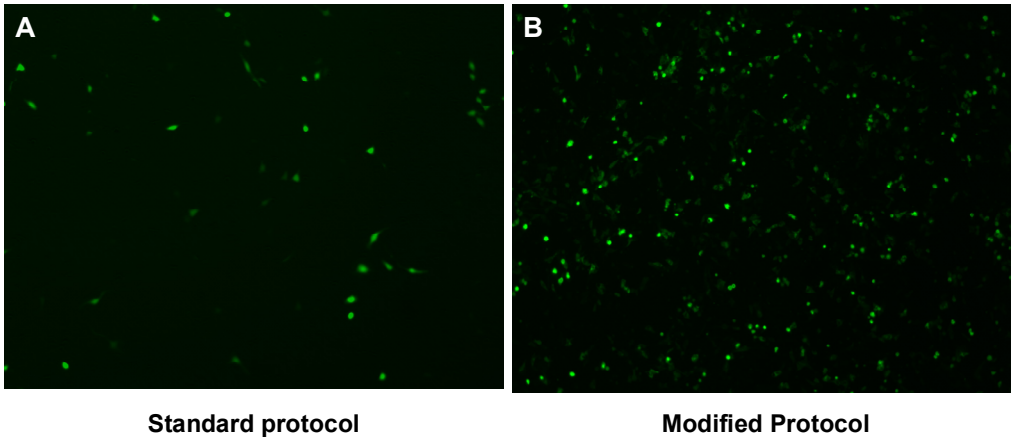


Figure 10 – Transfected SB-HSA cells Using Standard and Modified Protocol

SB-HSA cells were transfected by Lipofectamine 2000 with a GFP vector using both standard protocol and modified protocol. 48h after transfection, cells were photographed and compared.

3.4.4 Stable Transfection of SB-HSA cells

I next explored whether SB-HSA cells can be stably transfected. From preliminary antibiotic titration and optimal cell seeding density experiments, the optimal

transfection conditions were determined to be 250μg/ml G418 in growth medium and 60% confluent cell-seeding-density. These conditions were used to stably transfect SB-HSA cells with a luciferase vector containing a neomycin resistant gene, pGL4.18. After a week of antibiotic selection, a population of luciferase-expressing SB-HSA cells (SB-HSA-Luc) was observed and confirmed by luciferase assay (Figure 11).

Long-term culture of SB-HSA-Luc cells in either normal culture medium or G418-containing medium for 6 months revealed little difference in the levels of luciferase expression level (Figure 12), which indicates that the luciferase plasmid vector may have integrated into the genome of SB-HSA cells and a population of SB-HSA cells that constantly express luciferase has formed.

Since SB-HSA cells are also cancer cells, their use as delivery vehicle will raise safety concerns, I therefore tested if the proliferation of SB-HSA cells can be deactivated using irradiation without affecting their gene expression. After irradiation of SB-HSA-Luc cells, their proliferation was monitored for a week. Although a 5Gy dose did induce some cell death (Figure 13F-G) compared to untreated cells (Figure 13A-D), it did not stop the proliferation of SB-HSA-Luc cells (Figure 13G-H). 10Gy-dose completely inhibited SB-HSA cell proliferation and after 7 days (Figure 13L), those cells still expressed strong luciferase activity compared to normal SB-HSA cells (Figure 14). These data indicate that an adequate dose using 10Gy to irradiate SB-HSA-Luc cells could strongly inhibit their proliferation without abolishing their metabolic activity such as their luciferase expression and thus these cells could be applied as delivery vehicles (Table 10).

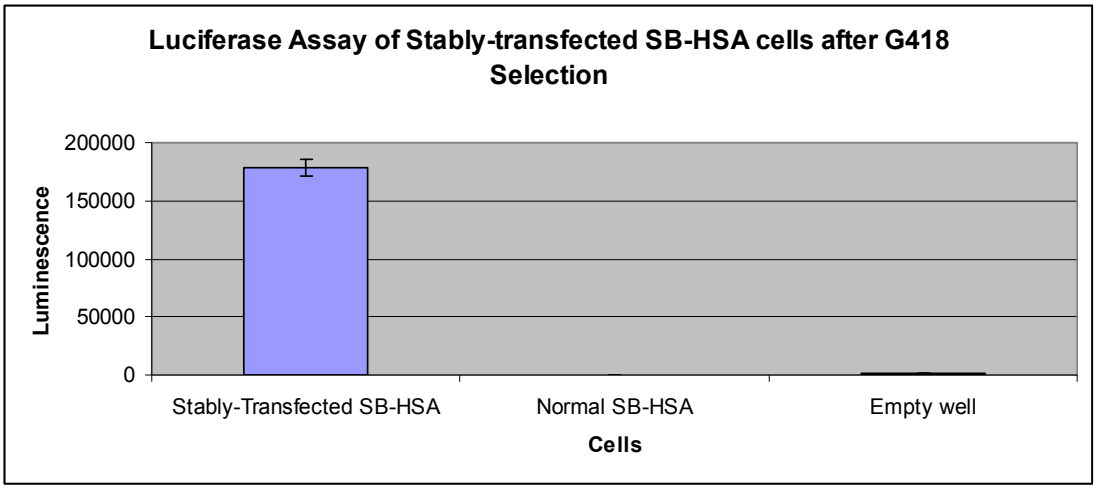


Figure 11 – Luciferase Assay of Stably-transfected SB-HSA Cells

After G418 antibiotic selection of stably-transfected SB-HSA cells, luciferase assay was performed along untransfected normal SB-HSA cells and empty wells as controls.

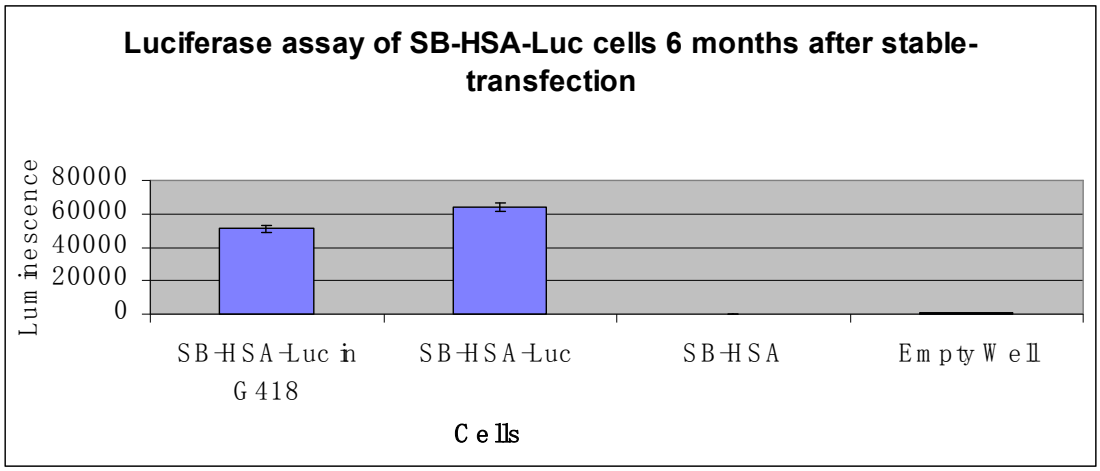


Figure 12 – Luciferase Assay of SB-HSA-Luc Cells in Medium with and without G418

After confirmation of stably-transfected SB-HSA cells, SB-HSA-Luc cell population was grown in two flasks with or without G418 antibiotics for 6 months. Luciferase assay was then performed on them along with untransfected normal SB-HSA cells and empty wells as controls.

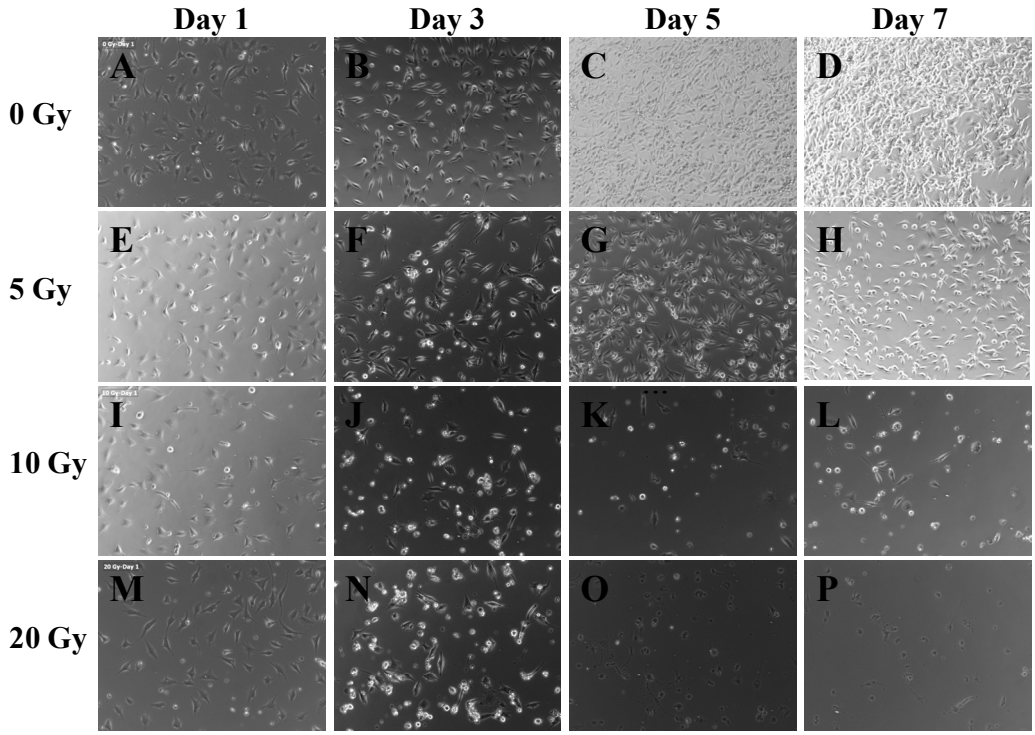


Figure 13 – Culture of SB-HSA-Luc Cells after Irradiation

SB-HSA-Luc cells were subjected to different doses of irradiation then continuously grown for 1 week. Photographs of cells were taken at Day 1, 3, 5 and 7.

	Day 1 – Day 7
0 Gy	+
5 Gy	+
10 Gy	-
20 Gy	-

Table 10- Qualitative Summary of Irradiation Results

Continued proliferation of cells that were subjected to certain dose is marked as + and ceased proliferation is marked as -

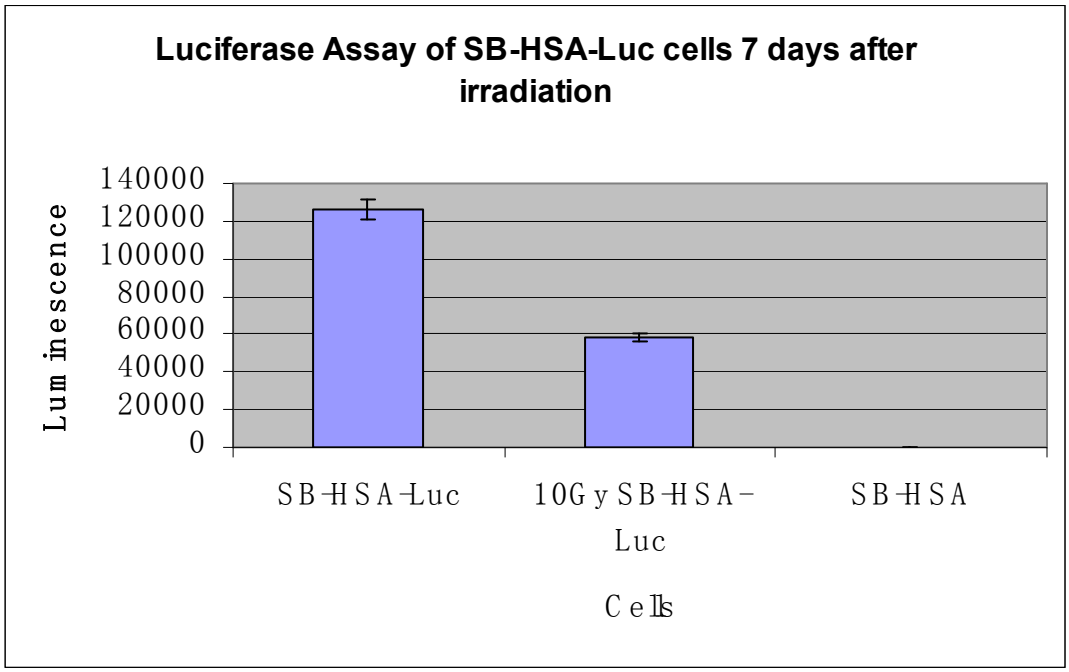


Figure 14 – Luciferase Assay of SB-HSA-Luc Cells after Irradiation

After 7 days culture of irradiated SB-HSA-Luc cells, luciferase assay was performed on 10Gy irradiated SB-HSA-Luc cells, on non-irradiated SB-HSA-Luc and untransfected normal SB-HSA cells.

3.4.5 Tissue Distribution of Intravenously Injected SB-HSA Cells in a Canine Tumour Mouse Model

To investigate if SB-HSA cells can be used as a potential cellular vehicle, SB-HSA cells were labelled with DiR-cell-membrane dye followed by i.v. administration into four mice containing canine tumour xenografts. 24 h after injection, a strong fluorescent signal was detected in the liver of these mice, indicating a strong liver uptake of these cells. A weaker fluorescent signal was found in the spleen, heart, hind leg, but not in the kidney. A strong fluorescent signal was also observed in the tumour site in any of the 4 mice and the signal level was comparable to the ones seen in other organs, indicating some SB-HSA cells did migrate to tumour site (Figure 15).

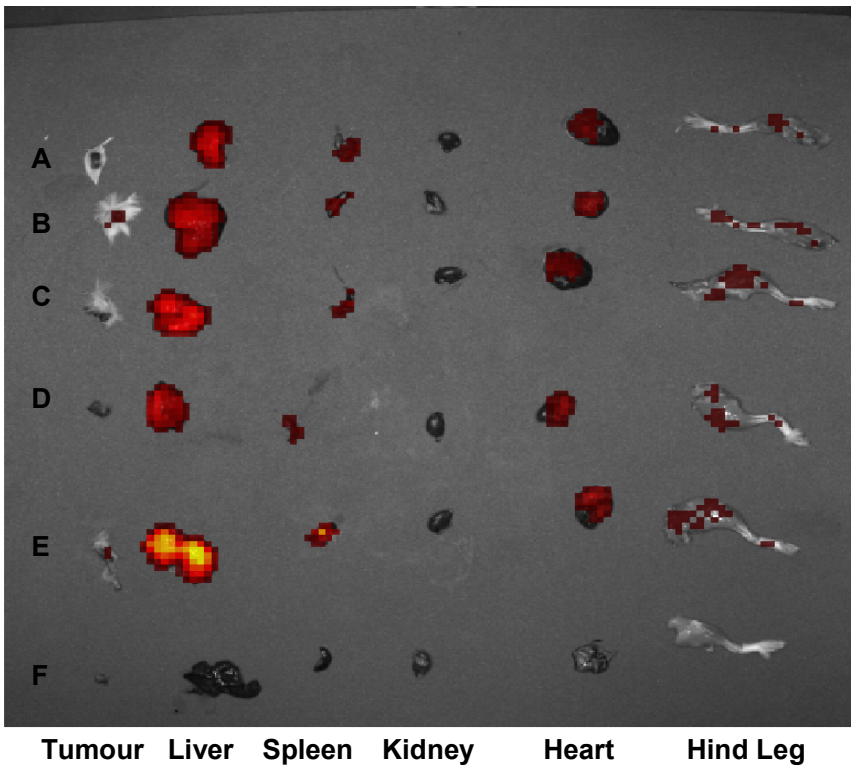


Figure 15 – *Ex vivo* Imaging of Labelled SB-HSA Cells after i.v. Injection

A-D, Labelled SB-HSA cells in cancer-bearing mouse

E, DiR dye alone in cancer-bearing mouse

F, Untreated cancer-bearing mouse

3.4.6 *In Vitro* Optimization of Transfection Efficiency of PPIG3 Dendrimers

To investigate the *in vitro* transfection nature of PPIG3 dendrimers, two canine tumour cell lines were transiently transfected with a GFP vector complexed to PPIG3 dendrimers (Table 11). Despite varying the input DNA amount from 1.5µg to 6µg, SB-HSA cells remained hard to transfect (Figure 16). Relatively higher transfection efficiency was achieved in D-17 cells (Figure 17). However, the overall transfection ability of PPIG3 found here is much lower and less efficient than that found using the Lipofectamine 2000 method.

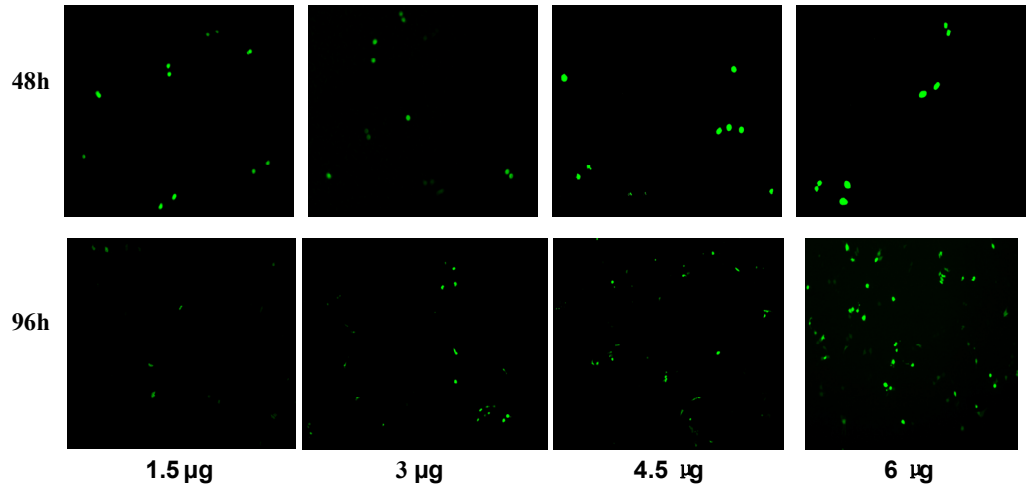


Figure 16 – Transfection of SB-HSA Cells by PPIG3 Dendrimers

SB-HSA cells were transfected with different amount of GFP plasmid complexed with PPIG3 dendrimers and photographed at 48h and 96h after transfection

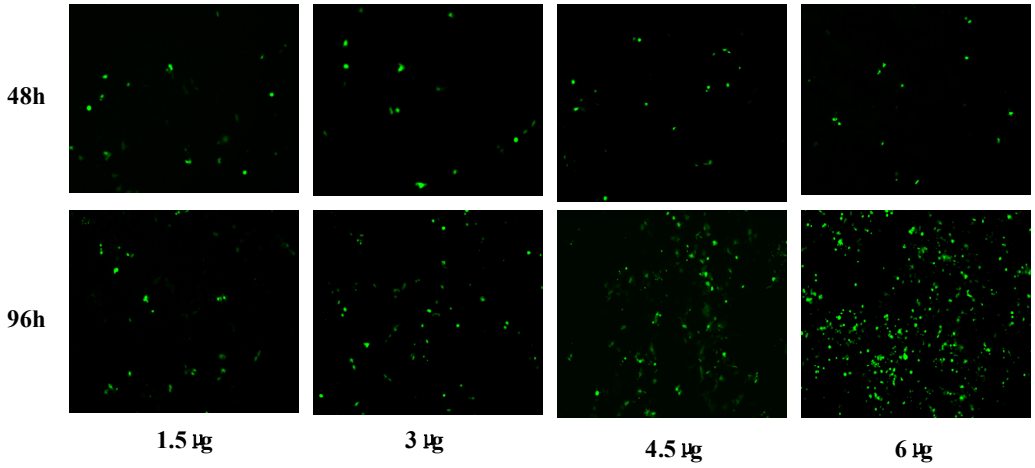


Figure 17 - Transfection of D-17 Cells by PPIG3 Dendrimers

D-17 cells were transfected with different amount of GFP plasmid complexed with PPIG3 dendrimers and photographed at 48h and 96h after transfection

	PPIG3 Dendrimers
SB-HSA Cells	+
D-17 Cells	+

Table 11- Qualitative Summary of Transfection by PPIG3 Dendrimers

Successful transfection using PPIG3 dendrimers was marked as +

3.4.7 Design and Construction of a siRNA Expression Vector Targeting cTR

Having achieved much higher transient and stable transfection in SB-HSA cells using a plasmid vector (Figure 10B, 11 and 12); I next decided to continue with the plasmid vector approach and to develop a DNA plasmid-based siRNA expression vector to target the canine telomerase RNA component.

The sequence of Human telomerase RNA component gene was obtained from the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/nuccore/NR_001566.1). I next used the BLAST algorithm to search the canine genome (*Canis lupus familiaris*) for this sequence to identify the predicted canine TR sequence (Figure 18). The sequence 5'-CGTCTAACCCTAACTGAGCAG-3' which was based on a previous study (Lund et al., 2008) was reported to target the template region of cTR and inhibit telomerase activity more consistently than other areas of the sequence. The insert sequence for siRNA plasmid was then designed (Figure 19) using the Ambion online design tool (http://www.ambion.com/techlib/misc/psilencer_converter.html), and the siRNA expression vector was then constructed according to the manufacturer's protocol.

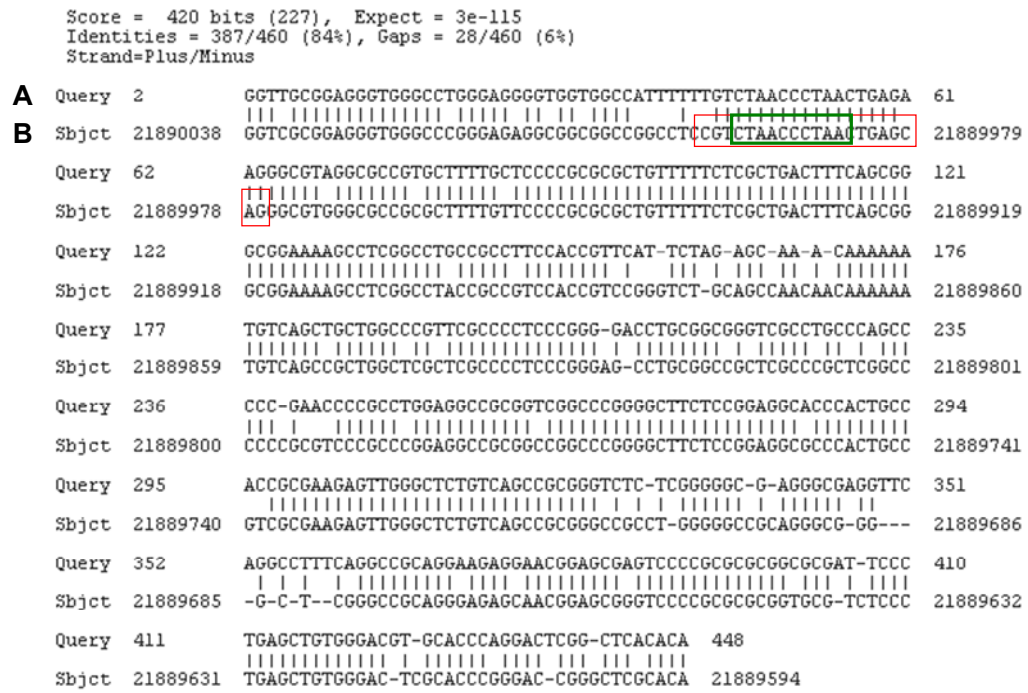


Figure 18 – Alignment of hTR Gene and Dog Genome

A, Human telomerase RNA component gene sequence

B, dog genome resources

Red box contains the target sequence of cTR gene and green box contains the template region of cTR.

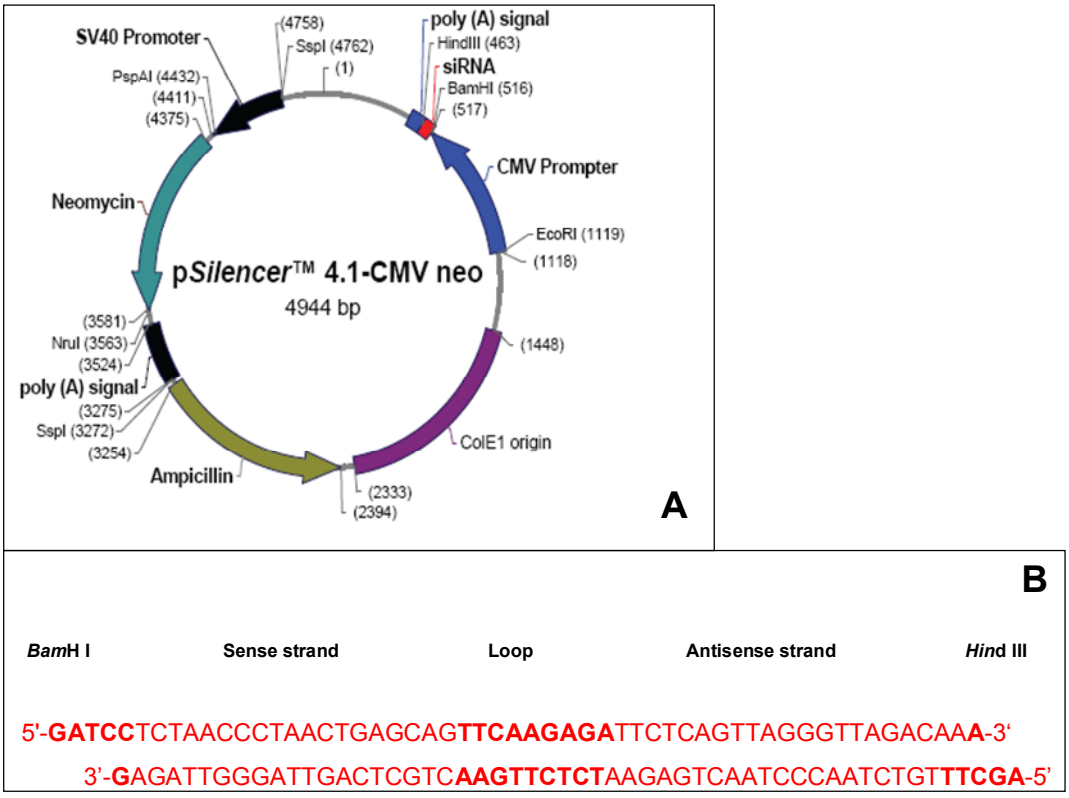


Figure 19 – Map of the siRNA Expression Vector and the Sequence of siRNA Insert

A, Map of the siRNA expression vector (adapted from pSilencer 4.1-CMV neo instruction manual). siRNA insert part is coloured in red.

B, The siRNA insert sequence targeting cTR

Highlighted sequence in the centre represents the loop sequence and highlighted sequence on both sides represents the restriction enzyme sites.

3.4.8 Optimizing the Transfection Efficiency of D-17 Cells

To analyse the gene-silencing effect of the siRNA vectors, I not only used canine cancer SB-HSA cells, but also chose another canine cancer cell type, D-17 cell, and the optimized transfection of this cell line using Lipofectamine 2000 modified protocol was demonstrated (Figure 20). A similar result (Table 12) was found for D-17 cells as was found in SB-HSA cells (Figure 10), in that the modified protocol of Lipofectamine 2000 strongly increased the transfection ability in these cells comparing with using standard protocol.

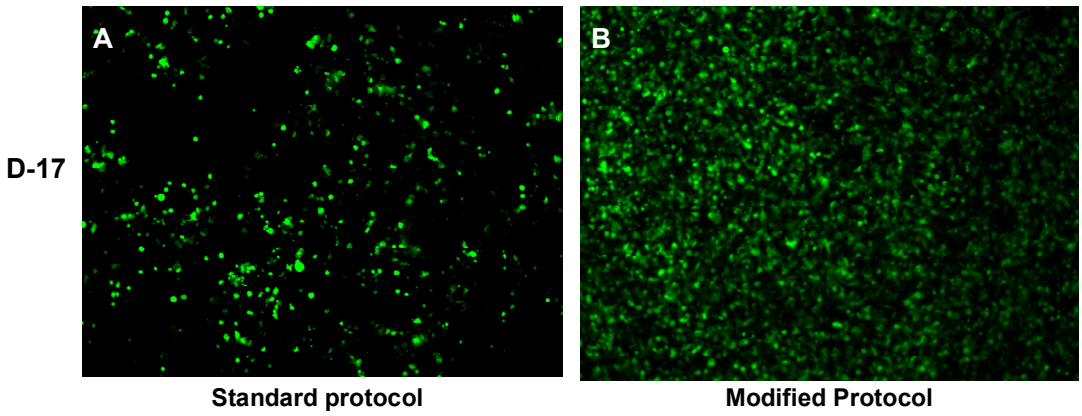


Figure 20 –Transient Transfection of D-17 Cells Using Standard and Modified Protocol

D-17 cells were transfected with a GFP plasmid using two different Lipofectamine 2000 protocols then photographs were taken 48h after transfection.

	Standard Protocol	Modified Protocol
SB-HSA Cells	+	++
D-17 Cells	+	++

Table 12- Summary of Improved Transfection

Successful standard protocol was marked as +

Any improved transfection result by using modified protocol of Lipofectamine 2000 was marked as ++

3.4.9 Design of a Duplex RT-PCR System for Analysing siRNA Effect

A duplex RT-PCR system was established to analyse the expression of cGAPDH and cTR in siRNA-transfected cells. Amplified cGAPDH corresponds to a 457bp band and cTR corresponds to a 157bp. These two bands appeared in the cDNA-added reaction groups only but not in the control reaction groups where cDNA was replaced with RNA or water (Figure 21).

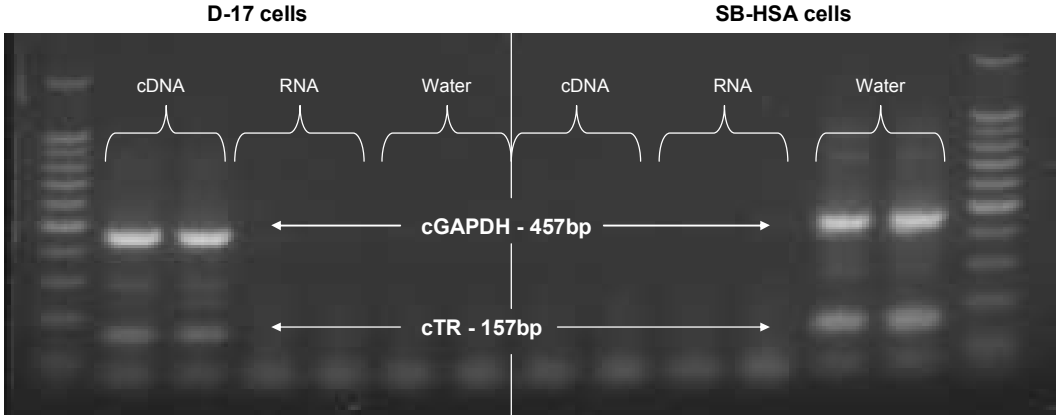


Figure 21 – Establishment of Duplex RT-PCR for cGAPDH and cTR

RT-PCR products were presented on gel in duplicates. DNA ladder was run on both sides of the gel.

cDNA, normal PCR reaction with cDNA in it.

RNA, PCR reaction with RNA replacing cDNA

Water, PCR reaction with water replacing cDNA

3.4.10 Demonstrating the Gene-silencing Ability of siRNA Expression Vector targeting hGAPDH

The siRNA expression vector kit came with a prevalidated siRNA insert ready to be ligated into the vector targeting human GAPDH. Thus I constructed this siRNA vector targeting hGAPDH and tested it on the human tumour cell line, H1299 to demonstrate its gene-silencing ability. Human GAPDH was shown to be significantly silenced 48h after transfection with the siRNA vector, compared with a scrambled siRNA negative control (Figure 22).

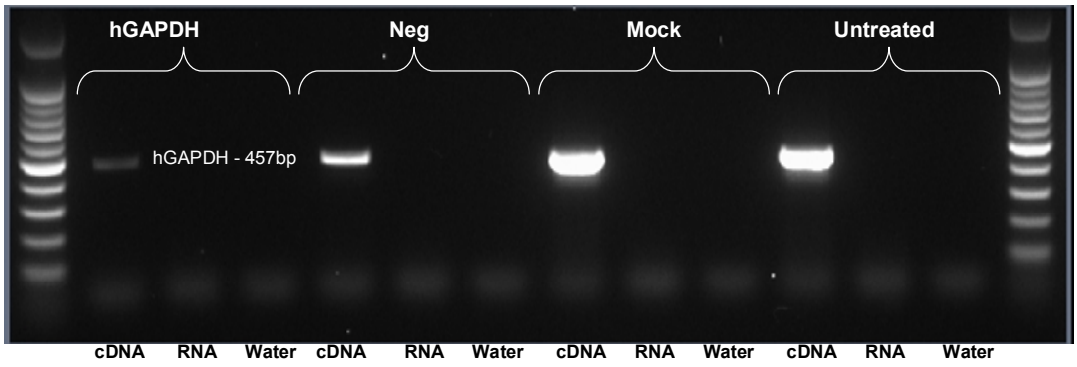


Figure 22 – Singleplex RT-PCR of hGAPDH-siRNA Vector-treated H1299 Cells

hGAPDH, hGAPDH-siRNA vector-treated H1299 cells

Neg, scrambled negative control siRNA vector-treated H1299 cells

Mock, mock transfection of H1299 cells

Untreated, untreated H1299 cells

DNA ladder was run on both sides of the gel.

CDNA, RNA and Water lane are the same as defined above (Figure 21)

3.4.11 Test of siRNA Targeting cTR in SB-HSA Cells

After demonstrating the gene-silencing ability of the vector itself, siRNA targeting c TR was transfected into canine cancer SB-HSA cells. 48h after transfection, results were analysed on the pre-established duplex RT-PCR system (Figure 21) for cGPAHD and cTR. No cTR gene-silencing was observed in SB-HSA cells following transfection of cTR siRNA (Figure 23).

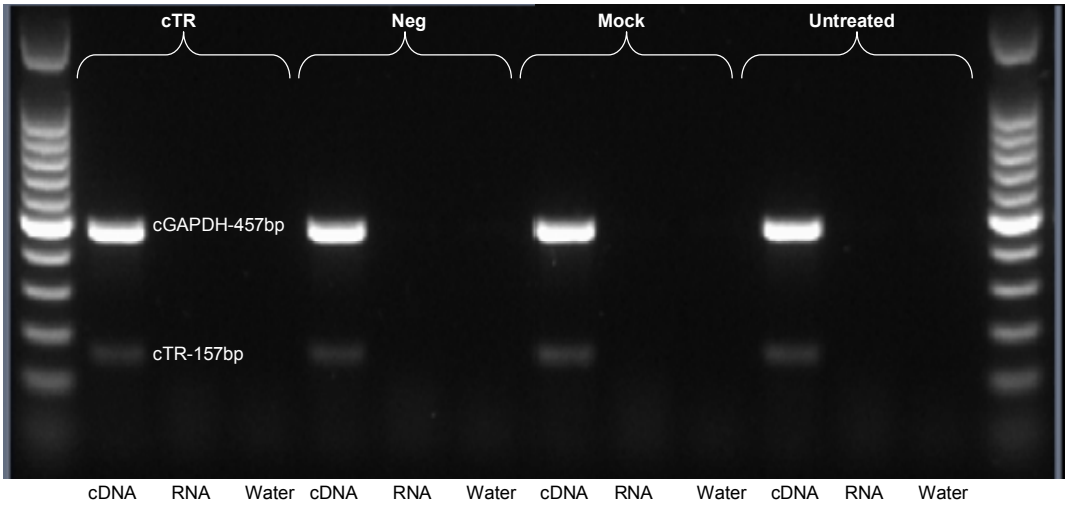


Figure 23 – Duplex RT-PCR of cTR-siRNA-treated SB-HSA Cells

cTR, cTR-siRNA treated-SB-HSA cells

DNA ladder was run on both sides of the gel.

Neg, Mock, Untreated, cDNA, RNA and water are the same as defined above (Figure 21 and 22)

3.4.12 Test of siRNA Targeting cTR in D-17 Cells

After testing on SB-HAS cells, the siRNA targeting cTR was tested on another canine cancer D-17 cells. No cTR gene silencing was observed in D-17 cells following transfection of cTR siRNA (Figure 24).

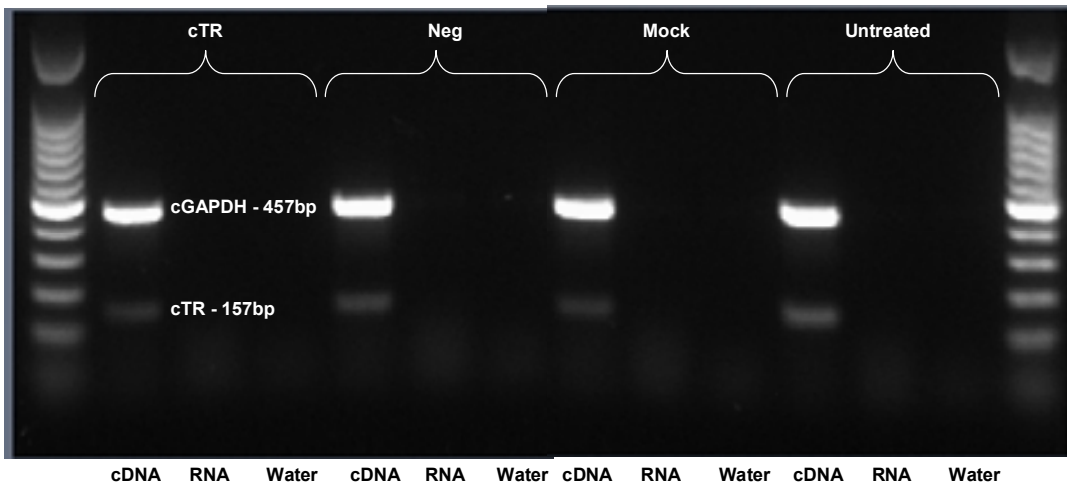


Figure 24 - Duplex RT-PCR of cTR-siRNA-treated D-17 Cells

DNA ladder were run on both sides of the gel.

cTR, Neg, Mock, Untreated, cDNA, RNA and water are the same as defined above (Figure 21, 22 and 23)

3.5 Discussion

Although we had already developed a viral siRNA vector capable of inhibiting canine telomerase *in vitro* (Lund et al., 2008), we still lacked an efficient delivery system for siRNA-based applications. As endothelial lineage cells have been reported to home to the tumour formation site, three endothelial cells, SB-HSA, HUVEC and EOMA cells were initially selected to begin our study.

As a high transfection efficiency is crucial for any siRNA-mediated gene silencing either *in vitro* or *in vivo*, I tried to achieve this by firstly exploring the ability of four different commercial transfection reagents to transfect these three different endothelial-type cell lines. Our initial experiments using the β-galactosidase reporter gene vector confirmed our expectation that endothelial cells are very hard to be transfected by most of the commercial reagents that were tried. However, SB-HSA cells transfected with Lipofectamine™ 2000 showed a relatively higher transfection efficiency compared with the other two cell types. This result prompted us to further optimize the transfection efficiency of SB-HSA cells. In order to perform consistent

and effective transfection experiment, I replaced the reporter gene β-galactosidase vector with a GFP vector, as I did encounter difficulties when handling β-galactosidase. Firstly, *in situ* detection of β-galactosidase within cells requires staining by x-gal before it can be visualised by microscopy and in order to gain consistent and effective staining, a fixation step before staining is also required. These two steps need multiple PBS washes to ensure that no inhibitors from either the cell medium or cell fixation process hinder the staining process. Moreover, I have found certain type of cells, such as 293FT, detached from the bottom of the plate very easily, sometimes even without PBS washing, which thus made it very difficult for us to do sufficient PBS washes while preventing these adherent cells detaching from the bottom of the plate. So the whole process of staining is very time-consuming. Losing cells during multiple steps of PBS washes could hinder us from obtaining a correct staining result. In addition, glutaraldehyde that was used in the fixation step is considered carcinogenic (Zeiger et al., 2005). In contrast, GFP emits green fluorescence upon exposure to UV or blue light and, unlike luciferase or β-galactosidase enzymes, does not require additional substrates for the enzymatic reaction (Martin and Murray, 2000), which thus saves significant assay time, especially in large experiments of transfection of multiple cell types using multiple transfection reagents.

Traditionally, most of the liposome-based reagents recommend plating cells 24h prior to transfection, which have limitations on transfection efficiency. Sometimes, despite varying the ratio of DNA:reagent, the cell-seeding density and/or the amount of DNA input, the improved is still very limited. I have shown here that by simply adding the DNA–Lipofectamine complex immediately following cell-plating on the day of transfection instead of plating cells 24h before transfection, I can significantly increase the transfection of both SB-HSA cells and D-17 cells to achieve at least double the transfection efficiency obtained using the standard protocol (Figure 10 and 20). This new transfection protocol, which can save at least 24h of the experiment time, is based on the so-called ‘reverse transfection’ and is mainly used for high throughput transfection assays (Martin and Murray, 2000, Dalby et al., 2004). With this enhanced transfection effect, not only an increased population of

transfected cells can be analysed for downstream applications, but also it gave us the idea of developing a plasmid vector-based siRNA to utilize this enhanced *in vitro* ability.

Cells generally can be transfected *in vitro* and *in vivo* using physical, viral or chemical approaches (Greco et al., 2002, Azzam and Domb, 2004, Kawakami et al., 2008). Physical methods, such as electroporation, has achieved some success in certain cell types and is cell cycle-independent, but may be toxic to some cell types and requires specialized equipment (Villemejane and Mir, 2009). Viral-vector-based transduction is reported to be efficient for gene transfer, especially for *in vivo* use (Work et al., 2004), but concerns have been raised over insertional mutagenesis, the immune response or viral infection that might be caused by these vectors (Glasgow et al., 2004, Kreppel and Kochanek, 2008). Generally higher safety measures are still required to use viral vector as transfection tools (Manilla et al., 2005, Raty et al., 2008). In contrast, transfection of cells using chemical methods is widely used, because the reagents are relatively simple to use, cheap and easy to handle (Douglas, 2008).

One of the most important features of an ideal cellular vehicle is that it should be easily modified to express foreign genes, either reporter genes for cell tracking or therapeutic genes for cancer therapy. Currently most studies modifying potential cellular vehicles have used viral methods for gene therapy-based studies (Chen et al., 2008). Also it was reported that using viral vectors to transfer therapeutic genes into endothelial progenitor cells could achieve as much as 80% transfection efficiency (Kealy et al., 2009). However, this method also bears the risk of introducing unexpected adverse or side-effects such as toxicity, immunogenicity (Marshall, 1999), mutagenesis and oncogenesis (Hacein-Bey-Abina et al., 2003, Kerbel et al., 2008). I transfected SB-HSA cells with pGL-4.18, a luciferase plasmid vector bearing an antibiotic resistant gene using chemical transfection methods followed by antibiotic selection. A luciferase assay demonstrated that transfected SB-HSA cells showed significant luciferase expression compared with untreated cells. I then cultured these cells in growth medium with or without antibiotics. Interestingly, six months after

transfection both cell populations were still strongly expressing luciferase indicating the plasmid may have integrated into the genome of cells. This observation suggests that non-viral methods can be used to transfer genes into cellular vehicles *ex vivo* to achieve long-term cancer gene therapy.

Even SB-HSA cells can be potential cellular vehicles for cancer gene therapy, although injecting malignant endothelial cells systemically increases the risk of initiating tumours *in vivo*. However, an adequate dose of irradiation should abolish the proliferation of malignant cells while allowing them to remain metabolically active. Thus, I investigated the irradiation response of luciferase-stably-transfected SB-HSA and SB-HSA-Luc cells. I can confirm that a high dose of 10Gy irradiation completely abolished the proliferation of SB-HSA cells compared with untreated cells, while still maintaining adequate expression of the reporter gene seven days after irradiation. This finding makes irradiation a possible way to abolish cancer cell proliferation without affecting its gene expression, and thus paves the way to utilize malignant cells, such as SB-HSA cells as cellular vehicles for cancer gene therapy.

Potential cellular vehicles with specific tumour-homing ability have always attracted great attention for cancer gene therapy. Our hypothesis is based on the fact that circulating endothelial lineage cells can migrate to solid tumour sites and contribute to tumour neovascularization to support tumour growth. However, this homing ability was reported to depend on the specific cancer type (Ruzinova et al., 2003, Kerbel et al., 2008), stage (Spring et al., 2005), grade (Li et al., 2004a), site and even mouse strain used (Duda et al., 2006, Madlambayan et al., 2009). In this study, I demonstrated that after intravenous administration of fluorescent DiR-dye labelled SB-HSA cells, one out of four cancer-bearing mice showed the localisation of SB-HSA cells to the subcutaneous tumour site. However the fluorescent signal also indicated that labelled SB-HSA cells also migrated to other major organs, such as the liver, spleen and bone marrow, but not the kidney. In the group injected with DiR only, I injected the same amount of DiR dye without cells into the tumour-bearing mice and a strong fluorescent signal was observed in all major organs, which indicates the non-specific binding of this dye to cell membranes *in vivo*. To avoid this

problem, after labelling cells, I washed cells with PBS 3 times each combined with 3 times centrifugation steps to eliminate any remaining dye from the cell medium. Although bioluminescence imaging bears certain advantages, it requires an additional step of administration of a substrate, luciferin, for the luciferase reaction *in vivo*. DiR membrane dye offers a simple solution and only requires an *ex vivo* cell-labelling step before intravenous administration (Kalchenko et al., 2006, Kerbel et al., 2008). Our finding is largely consistent with other reports regarding potential cellular vehicles that systemically delivered Mesenchymal stem cells which homed to major organs but not to kidney (Xiang et al., 2009). Although tumour localisation of SB-HSA cells was observed in one mouse out of four, its migration to other major organs would make it less efficient to deliver therapeutic genes to tumour sites. In the future, a second strategy is required to specifically direct these cells to any target organ or tissue for targeted cancer gene therapy. For systemically targeting all primary and metastatic tumours, strategies must be developed to increase specificity by avoiding major organ intake to reduce toxicity.

Recent encouraging *in vivo* reports of PPIG3 dendrimers have made it an attractive vector for cancer gene therapy. However, only a few studies report the transfection efficiency in a few human cell lines *in vitro* (Zinselmeyer et al., 2002, Hollins et al., 2004, Santhakumaran et al., 2004), let alone in any canine cells. In this study, for the first time I tested the transfection ability using PPIG3 dendrimers in two canine cancer cell lines, SB-HSA haemangiosarcoma cells and D-17 osteosarcoma cells. I kept the ratio of DNA to PPIG3 at 1:5 constantly in this study, as this has been confirmed to enable PPIG3 to pack DNA firmly and to form stable colloidal nanoparticles (Chisholm et al., 2009). However, I encountered a major difficulty to transfect SB-HSA cells, despite varying the DNA amount from 1.5 µg to 6 µg. As the reported transfection efficiency of endothelial cells is generally low (Hunt et al., 2010), this result was expected. In D-17 cells, better transfection was achieved with more than 50% GFP-positive cells 96 hours after transfection. Although I was able to transfect many more D-17 cells than SB-HSA cells using PPIG3 dendrimers, the efficiency using PPIG3 in these two cell lines was much lower than I demonstrated using Lipofectamine 2000 in the previous chapter. It was also reported that PPI

dendrimers including PPIG3 altered the endogenous expression of many genes in transfected cells (Omidi et al., 2005, Barar et al., 2009), However, although this could be cell dependant (Omidi and Barar, 2009), the intrinsic gene regulation mediated by PPI should be seriously considered when designing and developing vectors for cancer gene therapy. To utilize PPIG3 for cancer gene therapy in the canine subject, more studies are required to explore their *in vitro* effect on canine cells first.

After exploring two potential systemic delivery vehicles, I then designed and constructed a DNA plasmid-based siRNA expression vector that expresses shRNA in cells. It has been reported that the same vector construct targeting either housekeeping gene (Shan et al., 2009) or cancer-specific gene (Li et al., 2010) in human cancer cells can effectively knock down target gene expression, and a similar vector construct has been shown to downregulate hTERT gene effectively in a human cancer cell line (Zhang et al., 2006).

I also established a duplex semi-quantitative RT-PCR system to analyse target gene silencing and housekeeping gene expression and used proper controls of reaction groups with added RNA or NF-water replacing cDNA to rule out any general or genomic DNA contamination. I did try to build a quantitative real-time RT-PCR system but, because only a short partial sequence of cTR has been published, no probe/primer could be designed from Roche ProbeLibrary using that sequence and only one probe/primer pair was available to try from Applied Biosystems. Unfortunately, this probe/primer pair also amplified non-specific bands in RT-PCR despite trying several annealing temperatures.

In the siRNA experiments, I first used a siRNA expression construct targeting human GAPDH housekeeping gene in the human lung cancer H1299 cell line to explore the gene-silencing capability of this plasmid vector. I found that 48 hours after transfection, expression of the hGAPDH gene was significantly inhibited in the siRNA-vector-treated group, but was unaffected in both the mock transfection group and untreated group, which indicated the strong silencing ability of this vector.

However, I also observed some hGAPDH gene knockdown in cells transfected with a scrambled siRNA vector (Neg) that should not target any known human gene. This might be explained by the off-target effects of siRNA molecules, for which only a short region of sequence complementarity triggers the silencing effect (Jackson and Linsley, 2010). Although I tried our siRNA vector on two canine malignant cancer cell types, I did not observe any cTR gene knockdown. Currently there is no complete sequence of cTR gene published and only one short partial sequence of cTR is available online. I obtained the cTR sequence using BLAST algorithm to align the human TR sequence with the canine genome. Based on 19nt target sequence from previously-established siRNA viral vector (Lund et al., 2008), I then designed the new plasmid-based siRNA expression vector targeting the same region of cTR (21nt target sequence by extending the 19nt target sequence 1 nucleotide from both ends as well as replacing 1 nucleotide) according to the BLAST algorithm alignment between hTR and dog genome.

Transfection efficiency directly influences the gene silencing result. The canine SB-HSA cell line that I used in this study was derived from malignant endothelial cells (Akhtar et al., 2004). Although there is no direct report on the transfection efficiency of this cell line, there have been several reports regarding the transfection efficiency of human endothelial cells, either using chemical reagents (Sipehia and Martucci, 1995, Fife et al., 1998, Zeng et al., 2000, Kaiser and Toborek, 2001, Colombo et al., 2001) or electroporation (Teifel et al., 1997). So far, the best transfection efficiency achieved on human endothelial cells by chemical reagents is approximately 32%, and most of the reports indicated a transfection efficiency below 25% (Hunt et al., 2010). Our result demonstrated a strongly improved transfection in the canine endothelial type cell, SB-HSA, which should enhance the gene silencing effect in theory.

The transfection method also influences gene silencing efficiency. It was reported that Lipofectamine 2000 which I used in this study gave the best transfection efficiency for human endothelial cells when compared with other commercial chemical reagents (Hunt et al., 2010). Although one report showed greater than 90% transfection efficiency using viral vector on endothelial cells, this method is limited

by the investment of time, facilities, overall expense, and safety considerations (Wrighton et al., 1996, Merrick et al., 1996).

Due to the lack of publications on studies regarding canine telomerase (Colitz, 2008), I do not have a complete published sequence of canine telomerase RNA component. Thus I used the human TR gene to align the published dog genome as well as using the target sequence of a previously established siRNA viral vector. As the plasmid-based siRNA vector requires a 21nt target sequence, unlike the siRNA viral vector from Invitrogen which only requires a minimum of 19nt target sequence, it is reasonable to speculate that extending the previously-proven target sequence of cTR might have hindered the gene-silencing mediated by the induced RNAi. It is known that siRNA molecules targeting different sequences of the gene vary markedly in their effectiveness (Holen et al., 2002). There is also a possibility that secondary structures of the targeted mRNA region and/or binding of regulatory proteins might block the induced RNAi by our vector. It is also reasonable at this stage to question the vector's capability of mediating gene-silencing in canine cells, as currently the gene-silencing ability of this vector has only been demonstrated in human cells. Again, as canine telomerase inhibition mediated by siRNA is not as well documented as human telomerase, novel regulation of telomerase might also exist in dogs. It was reported that, unlike in humans, inhibition of murine TERT led to quick telomerase reactivation accompanied by upregulation of TERT and that this might be explained by the longer telomere present in murine cancer cells (Marie-Egyptienne et al., 2008, Sachsinger et al., 2001).

In this study I could not downregulate the cTR gene in canine malignant tumour cells by plasmid-based siRNA vector, but telomerase still remains an attractive anticancer target. In the future, more research needs to be done on canine telomerase to explore effective ways of inhibiting telomerase, as well as developing the dog as a model for studying human telomerase. Further, not only is an effective way to inhibit telomerase needed, but a systemic delivery method is also crucial for its successful anticancer therapeutic application.

3.6 Conclusion

In this study, I initially explored two novel delivery systems which could be potentially utilized to deliver therapeutic genes systemically for treating cancer. As a starting point, I explored the transfection efficiency of three endothelial cell lines. Then I further optimised the transfection efficiency of SB-HSA cells using a chemical transfection reagent. Canine SB-HSA cells, as I found in our *in vitro* experiments, can be manipulated by chemical transfection methods for gene transfer short-term or long term. *In vivo*, although I did observe some cell migration into the tumour sites in one of the mice, labelled SB-HSA cells showed a wide tissue distribution to all major organs. Thus, SB-HSA cells might be difficult to utilize as a systemic delivery vehicle at this stage and this approach therefore requires further evaluation. Although the PPIG3 dendrimer was found to passively accumulate specifically in tumour sites *in vivo*, the *in vitro* transfection ability in canine cells were found to be relatively low in this study. Thus, this approach needs further study to improve its transfection ability as well as to understand the nature of its specific tumour accumulation *in vivo*. While numerous promising delivery systems have been developed for cancer gene therapy, most of the findings have not passed the proof-of-principle stage *in vitro* or *in vivo*. Each vehicle bears both advantages and drawbacks, thus harnessing their individual advantages to target different cancers, as well as using them in combination, may be the future solution for cancers treatment. After exploring two novel delivery systems, I then designed and constructed a plasmid-based siRNA expression vector targeting canine TR gene to inhibit telomerase. However, I did not observe any gene knockdown of cTR gene using the siRNA vectors. Although human telomerase is a promising anticancer target that has been intensively studied, canine telomerase is still poorly documented. This study indicated that using siRNA at least plasmid-based siRNA vector to target canine telomerase needs to be validated by more research in the future.

CHAPTER 4

THE INVESTIGATION OF CIRCULATING TELOMERASE REVERSE TRANSCRIPTASE MRNA IN CANINE CANCER PATIENT SERUM

4.1 Chapter Abstract

Despite the current advances in anticancer therapy, cancer still remains a high morbidity and mortality disease in both the human and canine fields. Finding a non- or minimum-invasive method to detect cancer has become the priority in the cancer research field worldwide. Although there have been many reports regarding the potential diagnostic use of hTERT mRNA found in the circulation of human cancer patients, the exact mechanism behind it is still poorly understood, and there is currently no investigation in the canine area.

To utilize the many canine cancer cases in the Hospital for Small Animals, I have investigated the existence of circulating TERT mRNA in the serum of canine cancer patients and its potential use in canine cancer diagnostics. Three different methods have been developed successfully to extract circulating RNA from as little as 100 µl canine serum, and both conventional RT-PCR and quantitative real-time RT-PCR systems have been established to analyse the levels of circulating RNA.

Among 36 canine serum samples that were randomly collected from the oncology department, 18s rRNA was present in all samples that were tested and cGAPDH mRNA was detectable in 26 serum samples. However, cTERT mRNA was only detected in 1 of these samples.

Although this project is only a pilot study of circulating RNA in the canine cancer field, the results have shown that more research needs to be done regarding the general mechanism behind circulating RNA before circulating cTERT can be used as a canine cancer diagnostic marker.

4.2 Introduction

According to the definition of 'biomarker' in the dictionary of cancer terms published online by the National Cancer Institute (www.cancer.gov/dictionary), it is a biological molecule found in blood, other body fluids or tissues that is a sign of a normal or abnormal process, or of a condition or disease. An effective biomarker for cancer should facilitate cancer detection in clinical or, preferably, sub-clinically

affected patients, and may also help to improve the clinical outcome of cancer patients. Thus, the identification of biomarkers that distinguish cancer patients from normal individuals is of great interest in both the human and veterinary fields. Although some research has been conducted in veterinary oncology to identify cancer biomarkers using advanced techniques such as proteomics (McCaw et al., 2007), there is still a long way to go before any candidate can be utilised in cancer clinics (Henry, 2010). Circulating RNA found in a number of human cancer patients provides a new way to discover biomarkers to detect cancer and, together with the universal tumour marker telomerase, if found to be valid, can be applied not only in cancer diagnosis but possibly also in prognosis, cancer staging or in monitoring the response to therapy. Despite there having been many studies linking circulating RNA with human cancers as a clinical marker, there has been no research done in the canine cancer field. Utilising the resources of our Oncology Department in the Veterinary Teaching Hospital, I aimed to answer two questions in this pilot study.

1. Is RNA present in the serum of canine cancer patients?
    - Three methods were investigated for total RNA extraction from serum of canine cancer patients.
2. Is amplifiable mRNA present in the serum of canine patients?
    - I investigated whether 18S rRNA and GAPDH mRNA could be detected in serum from canine cancer patients by conventional and/or real-time PCR.
3. Does TERT mRNA exist in the serum of canine cancer patients?
    - I investigated whether TERT mRNA can be detected in serum from canine cancer patients

4.3 Materials and Methods

4.3.1 Blood Sample Processing

Canine blood samples were randomly collected by veterinarians from canine cancer patients in the Oncology Department of Hospital for Small Animals at the University of Edinburgh. Samples were kept on ice and immediately processed to separate

serum from blood cells. To obtain a cell-free serum, I used 3-spin protocol. Blood samples were centrifuged at 1,000 x g for 10min, then at 1,500 x g for 10min, and finally at 3,000 x g for 10min at 4˚C. The serum was harvested at each step and taken forward to the next step. Purified serum was then stored at - 70˚C.

4.3.2 Total RNA Isolation from Serum

As total RNA from serum was reported to be extremely low (Fleischhacker and Schmidt, 2007), and presumably the amount of mRNA was even lower, three different methods were tried to optimize mRNA recovery.

4.3.2.1 The SV Total RNA Isolation System

The first method I will describe is the SV Total RNA Isolation System (Promega, UK). All components mentioned below were provided by the kit unless otherwise stated. Serum samples were first thawed on ice. In the meantime, NF-water (provided in the kit) was added to a vial of lypholized DNase I, and 200µl β-mercaptoethanol (Sigma, UK) was added to 10ml RNA Lysis buffer. 8ml and 20ml 95% ethanol were added to the DNase Stop solution and to the RNA Wash solution, respectively. The manufacturer's protocol was followed with only slight modifications (Chen et al., 2000). 175µl RNA Lysis buffer was directly added to 100µl serum, and then thoroughly mixed by inversion. 350µl RNA Dilution buffer was then added to the lysate before placing in a heating block at 70ºC for 3 min. The lysate was then centrifuged at 14,000 x g for 10min. After centrifugation, the supernatant was transferred to a new microcentrifuge tube and 200µl 95% ethanol was added and mixed by pipetting 3–4 times. The mixture was transferred to the Spin Column Assembly and centrifuged at 14,000 x g for 1min. After discarding the flow-through, 600µl RNA Wash solution was added into the spin column, which was then centrifuged at 14,000 x g for 1min. The column collection tube was emptied, then the DNase incubation mix was prepared by combining 40µl Yellow Core buffer, 5µl 90mM $MnCl_2$ and 5µl of DNase I enzyme. 50µl of the DNase mix was then applied to the membrane of the column. After incubating for 15min at room temperature, 200µl DNase Stop solution was applied to the membrane then centrifuged at 14,000

x g for 1min. 600µl RNA Wash solution was applied to the column for a second time followed by 14,000 x g centrifugation for 1min. After empting the collection tube, another 250µl RNA Wash solution was added to the spin basket and it was then centrifuged for 2min at 14,000 x g. Finally, the spin column was transferred to an elution tube 30µl NF-Water was added and it was then centrifuged at 14,000 for 1min.

4.3.2.2 The High Pure Viral RNA Kit

The second method I will describe is the High Pure Viral RNA Kit (Roche, UK). This system was designed for processing 200µl serum samples, but it can be used to process up to 600µl serum at a time if all components are increased proportionately and loaded into the filter tubes three times. All components mentioned below were provided by the kit unless otherwise stated. 600µl serum samples and 1.2ml Carrier RNA-supplemented Working solution was added to a clean tube and mixed well. The mixture was then loaded onto a High Pure Filter Tube several times and centrifuged at 8,000 x g for 15s after each addition. After discarding the flow-through, the filter tube was transferred to a new collection tube. 500µl Inhibitor Removal buffer was then added to the tube assembly and centrifuged at 8,000 x g for 1min. This process was repeated twice. After this, 450µl Wash buffer was added to the tube assembly three times and centrifuged at 8,000 x g for 1min after each addition. The filter tube was then transferred to a new collection tube and washed three times with 450µl Wash Buffer, with centrifugation at 8,000 x g for 1min after each addition. After empting the collection tube, the assembly was centrifuged at 12,000 x g for 10s to remove any residual Wash buffer and this step was repeated three times. The filter tube was then placed into a clean microcentrifuge tube and 30µl (50µl for 200403,300664 and 172229 for tests in both conventional and real-time PCR) Elution buffer was applied to the assembly and centrifuged at 8,000 x g for 1min to elute the RNA.

4.3.2.3 The TRI Reagent® BD

The third method I will describe is the TRI Reagent® BD (Sigma, UK). For using the

TRI BD Reagent, 250µl serum was added to 750µl TRI Reagent® BD and mixed thoroughly by vortexing. After incubation at room temperature for 5min, 100µl 1-bromo-3-chloropropane was added to TRI Reagent-serum mix. The tube was then covered and shaken vigorously for 15s. After 5min incubation at room temperature, the mixture was centrifuged at 12,000 x g for 15min at 4ºC. Centrifugation resulted in 3 phases of the mixture. The aqueous phase containing the RNA was carefully transferred to a new tube and 0.5ml isopropanol was added to the tube containing RNA. After 10 min incubation, the mixture was centrifuged at 12,000 x g for 8min at 4˚C. The centrifugation step resulted in a RNA pellet forming on the side and bottom of the tube. The supernatant was carefully removed and 1ml 75% ethanol was added in to precipitate RNA. The sample was vortexed and centrifuged at 7,500 x g for 5min at 4ºC. The supernatant was removed and the RNA pellet was briefly dried for 5–10 min, then 30µl NF-water was used to dissolve RNA. This method can be scaled up if more than 250µl serum needs to be processed.

RNA samples obtained from each of the above methods were either stored at -70˚C or treated with DNase for immediate use in downstream applications.

4.3.3 cDNA Synthesis from Serum RNA

4.3.3.1 cDNA Synthesis from Serum RNA for RT-PCR

Due to the very low amount of RNA in serum, all cDNA synthesis for downstream RT-PCR was conducted using Sensiscript RT Kit (Qiagen, UK). The recipe for a single reaction is given below (Table 13). Mixed components were then incubated at 37ºC for 60min. Synthesized cDNA can be stored in -20ºC or used in downstream RT-PCR directly.

Component	Amount (µl)
10x Buffer RT	2
dNTP Mix (5mM each dNTP)	2
Random Primers, 0.5µg/µl	1
RNase inhibitor (10 units/µl)	1
Sensiscript Reverse Transcriptase	1
Total RNA from Serum	« 13
NF-water (If needed)	To a final volume of 20

Table 13 – cDNA Synthesis from Serum RNA for Conventional PCR

All components mentioned above in the table were provided by the kit unless otherwise stated. As much as possible serum RNA was added into the reactions to generate the cDNA for downstream PCR detection, as long as enough RNA was saved for the control groups of PCR.

4.3.3.2 cDNA Synthesis from Serum RNA for Real-time PCR

For real-time PCR, Transcriptor High Fidelity cDNA Synthesis Kit was used to match the LightCycler 480 system. As much as 9.4µl extracted serum RNA was first mixed with 2µl of 600pmol/µl Random Hexamer Primer to make a 11.4µl RNA-primer mix. The mixture was then denatured in a heating block at 65˚C for 10min followed by immediate cooling on ice. The full reaction mixture was then assembled according to the table below (Table 14). It was then incubated at 29˚C for 10min followed by 60min at 48˚C. Finally, the reverse transcriptase was inactivated at 85˚C for 5min. Synthesized cDNA can be used in downstream real-time PCR directly or stored at -20˚C.

Component	Amount (µl)
Serum RNA and Random Hexamer Mix	11.4
Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer 5X	4
Protector Rnase Inhibitor 40U/µl	0.5
Deoxynucleotide Mix, 10mM each	2
Dithiothreitol (DTT)	1
Transcriptor High Fidelity Reverse Transcriptase	1.1

Table 14 – cDNA Synthesis from Serum RNA for Real-time PCR

All components mentioned above were provided by the kit unless otherwise stated. As much as possible serum RNA was added into the reaction to generate the cDNA for downstream PCR detection, as long as enough RNA was saved for the control groups of PCR.

4.3.4 Conventional RT-PCR for Serum RNA

Conventional RT-PCR was performed using the GoTaq® PCR Core System to detect cGAPDH and cTERT cDNAs. The recipe and primer sets are shown below (table 15 and 16) and cGAPDH primers were the same set that was used in the previous chapter (Table 7). The PCR products were run out on a 1% agarose gel, and the cTERT PCR product corresponded to a 279bp band. The same programme of PCR was run according to the previous chapter (3.3.8), and primer-dependent temperature was applied to the annealing temperature.

PCR products were then subjected to DNA electrophoresis and corresponding bands for cTERT and cGAPDH were excised using the same method mentioned in the previous chapter (3.3.7.2) for sequencing confirmation.

Component	Amount (µl)
cDNA / RNA / Water	5 / RNA / Water
dNTP, 10mM	1
$MgCl_2$, 25mM	4
5X Go Taxi Buffer	10
cTERT (cGAPDH) Forward Primer, 100pmol/µl	0.25
cTERT (cGAPDH) Reverse Primer, 100pmol/µl	0.25
GoTaq® DNA Polymerase, 5units/µl	0.25
NF-water	To a final volume of 50

Table 15 – Conventional PCR of cGAPDH or cTERT in Serum RNA

All components mentioned above in the table were provided by the kit unless otherwise stated. This set of PCR reactions used 5µl cDNA solution and 0.25µl PCR primers in order to detect the target gene in serum RNA.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Primer-dependant Temperature (˚C)
cTERT	TACCTGCCCAACACGGTAAC	GCGTGTGGGTCTGAGGTC	58

Table 16 – The Primer Set in Conventional PCR for Serum RNA

cGAPDH primers were the same set that was used in the previous chapter (Table 7)

4.3.5 Real-time PCR for Serum RNA

Duplex real-time PCR of serum RNA was performed analysing cTERT or cGAPDH mRNAs with 18S ribosomal RNA as control. cTERT and cGAPDH primers were labelled with the FAM dye and 18S rRNA was labelled with the VIC dye. All real-time PCR were done using LightCycler® 480 Probes Master (Roche, United Kingdom) on a LightCycler® 480 System. Real-time probes and primers were designed using the Universal ProbeLibrary System. Real-time PCR was conducted using 96-well plate in 10µl reaction volumes.

The efficiency of all Primers (Table 18) was tested first by running duplex real-time PCR of cGAPDH or cTERT with 18S rRNA using serially diluted cDNA samples (Table 17). Real-time PCR conditions were: an initial pre-incubation step of 95ºC for

10min; followed by 50 cycles of the amplification step, consisting of 95˚C for 10s, then the primer-dependent annealing temperature (T_m) for 30 s, followed by 72˚C for 1s; then with a cooling step of 40˚C for 10s. For each primer pair, the T_m is based on the mean annealing temperature of each primer pair. Control reactions included RNA or water to replace cDNA in the reaction, and all reactions were done in triplicate.

Component	Amount (µl)
LightCycler® 480 Probes Master Mix 10µM	5
Probes and Primers Mix 50µM	0.15
Probes and Primers Mix 50µM (2nd set)	0.15
cDNA / RNA / Water	4.7

Table 17 – Reaction Recipe for Duplex Real-time PCR of cGAPDH/cTERT with 18S rRNA

The above recipe is for one reaction. LightCycler® 480 Probes Master Mix was purchased from Roche and was ready to use.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Primer-dependant Temperature (˚C)
Eukaryotic 18s rRNA	Eukaryotic 18s r RNA Endogenous Control (VIC Probe)		52
cTERT	TGACGTGGA AGATGAAGGTG	TCTCTCCGA CGGTGTTCAG	57
cGAPDH	AATGTATCAGTT GTGGATCTGACC	GCTTCACTACCT TCTTGATGTCG	59

Table 18 – Primer Sets for Duplex Real-time PCR Analysing Serum RNA

Eukaryotic 18S rRNA was purchased from Applied Biosystems and was ready to use in the reaction. The primer sequence is proprietary.

4.4 Results

4.4.1 Collection of Blood Samples

From June to December 2009, 36 canine blood samples from the oncology department of Hospital for Small Animals were randomly collected. All the dogs sampled were suspected cases of neoplasia. This study was ethically approved and informed consent was obtained from each patient's owner.

4.4.2 Processing of Blood Samples

The sample processing is summarized in the diagram below (Figure 25). 36 serum samples ranging in volume from 100µl to 3.5ml were harvested and immediately stored at -70 ˚C. Three different methods for isolating total RNA from serum of canine cancer patients were tried, depending on the volume of serum in each sample. First, total RNA was successfully isolated using the Promega SV total RNA isolation system, but this system could only process up to 100µl serum sample at a time (Chen et al., 2000). The Roche High Pure Viral RNA kit protocol was also used to isolate total RNA successfully and could process up to 600µl serum at a time. Lastly, all serum samples larger than 600µl were processed using the Sigma TRI BD Reagent method.

One serum sample that was subjected to the Promega SV total RNA isolation method produced, after DNase treatment, 8.34ng/µl and the ratio of absorbance at 260nm and 280nm was 1.33. 16 samples that were subjected to Roche High Pure Viral RNA kit gave a yield ranging from 1.42ng/µl to 4.85ng/µl and the 260/280 ratio was from 0.54 to 1.5. Using TRI BD reagent, I extracted total RNA from nineteen samples where the yield ranged from 17.47ng/µl to 600.91ng/µl and the 260/280 ratio was from 0.59 to 2.08.

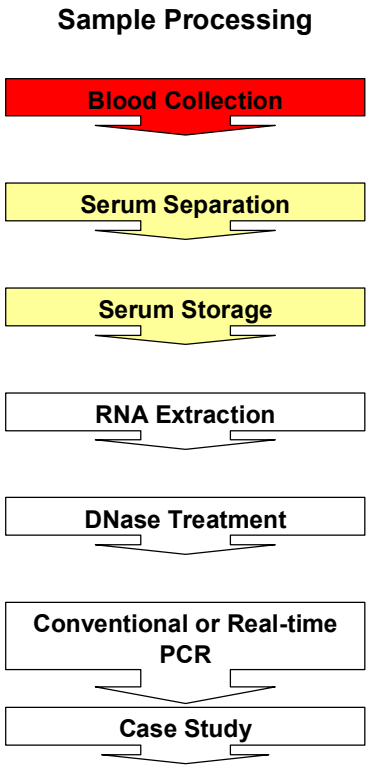


Figure 25 – Workflow of Blood Sample Processing

Experiment started with blood sample collection from the clinic followed immediately by serum separation and then the serum was stored at -70˚C. After extraction of total RNA from serum samples and DNase treatment, they were subjected to conventional and/or real-time PCR. At the end of the experiment case files relating to each sample were accessed and studied.

4.4.3 Establishment of Conventional RT-PCR

A conventional singleplex RT-PCR method was established to analyse cTERT or cGAPDH mRNA levels in both serum and cells (Figure 26). Canine malignant SB-HSA cells were used as cTERT and cGAPDH positive controls in this system. A band of 457bp cGAPDH and 278bp cTERT could be amplified from cDNA reaction group with cDNA synthesised from total RNA of SB-HSA cells compared with no band amplified from reaction groups with added RNA or water replacing cDNA.

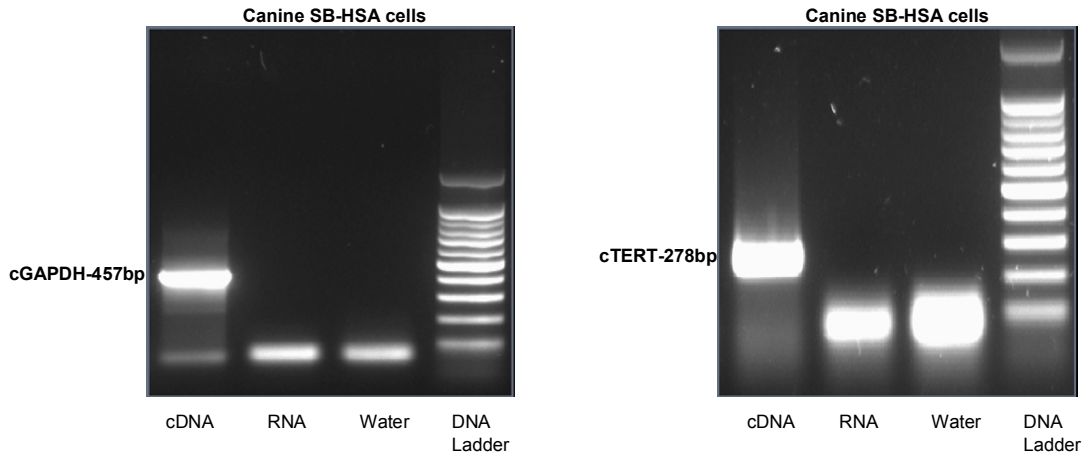


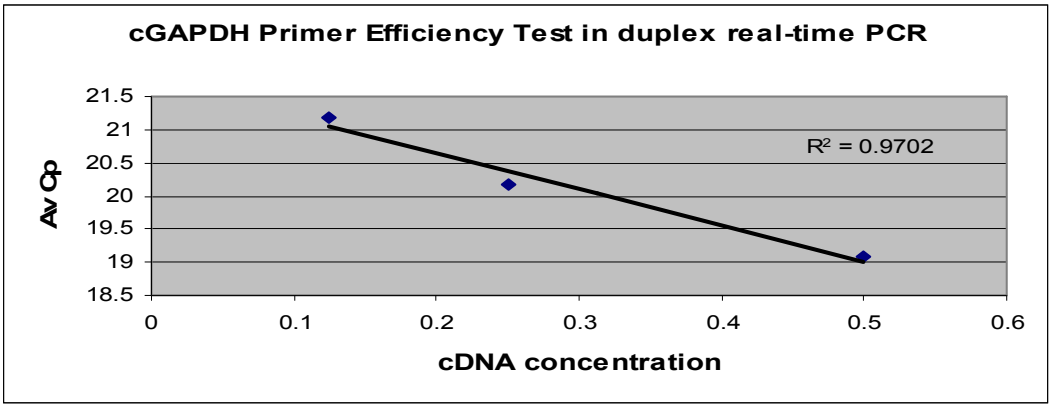
Figure 26 – Establishment of Conventional PCR for cTERT and cGAPDH Expression Analyzing Serum RNA

DNA ladders were run on the right side of both gels.
CDNA, RNA and Water lane are the same as mentioned earlier (Figure 21)

4.4.4 Establishment of Duplex Real-time PCR

Two duplex quantitative real-time RT-PCR systems were designed to analyse cTERT or cGAPDH mRNA expression with 18S rRNA levels as control in both serum samples and control SB-HSA cells. Canine malignant SB-HSA cells were used as positive controls for cTERT, cGAPDH mRNA and 18S rRNA amplification reactions. The efficiency of all 3 sets of primers was determined using serial dilutions of input cDNA. For duplex cGAPDH and 18S real-time PCR system, the R^2 is 0.9702 and 0.9751 (Figure 27) and for duplex cTERT and 18S real-time PCR system, the R^2 is 0.9887 and 0.9943 (Figure 28) which all indicated two efficient duplex real-time PCR systems.

A



B

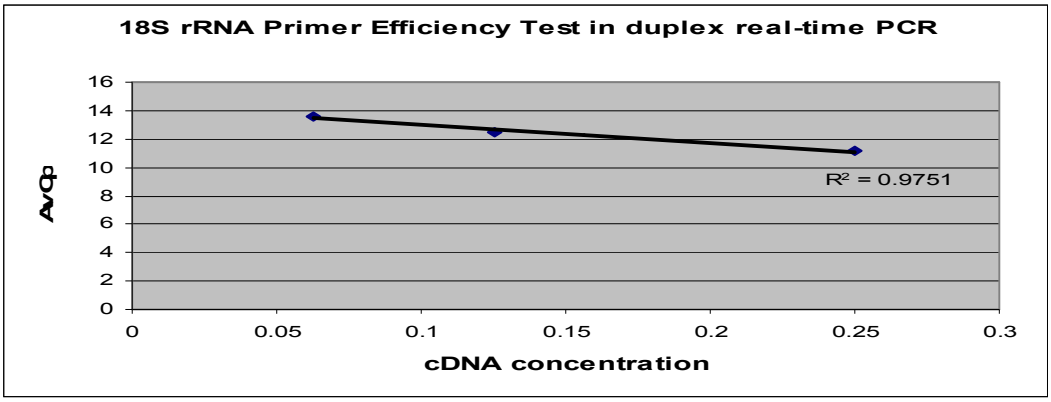


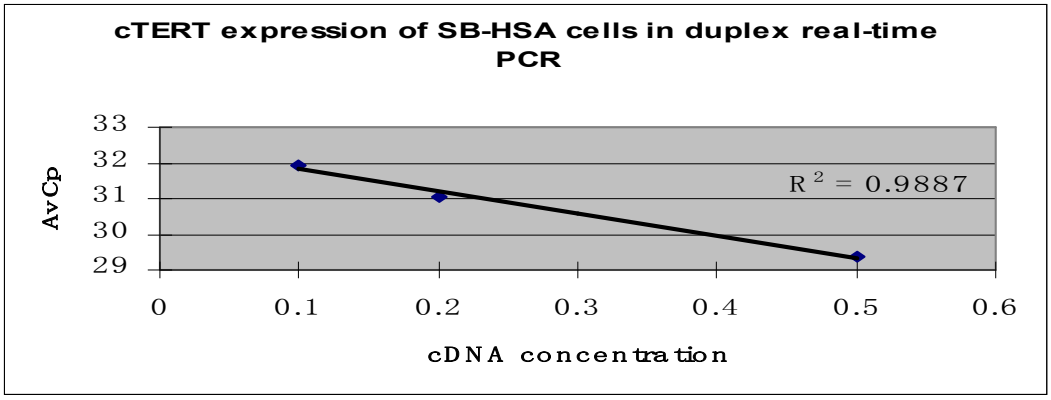
Figure 27 – Primer Efficiency Test of Duplex Real-time PCR of cGAPDH mRNA and 18S rRNA

A, The primer efficiency of cGAPDH mRNA within the system

B, The primer efficiency of 18S rRNA within the system

Av Cp stands for average Cp value

A



B

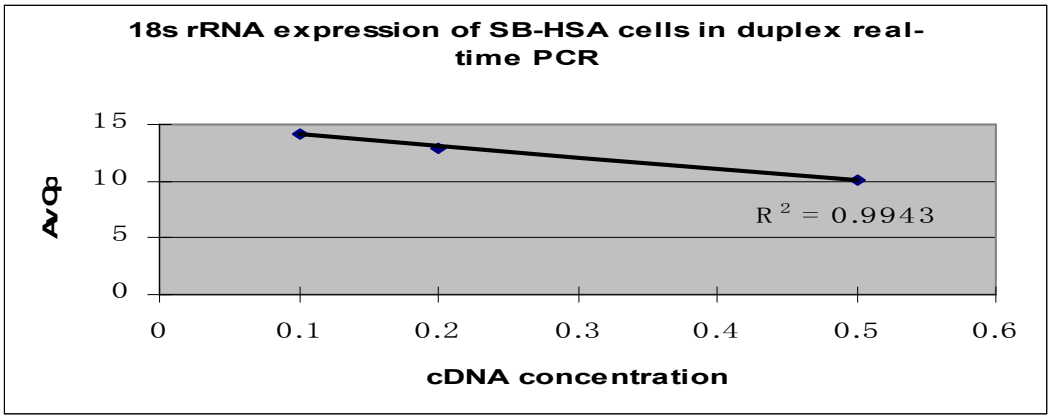


Figure 28 – Primer Efficiency Test of Duplex Real-time PCR of cTERT mRNA and 18S rRNA

A, The primer efficiency of cTERT within the system

B, The primer efficiency of 18S within the system

Av Cp stands for average Cp value

4.4.5 Result of Samples subjected to Conventional PCR Test

In the beginning of the test, I randomly analysed 13 samples using conventional RT-PCR and after this I started using real-time PCR system to analyse the remaining 23 samples. For the 13 serum samples subject to conventional PCR analysis, 6 samples were positive for the expression of the cGAPDH gene, and within these only 1 sample was positive for canine TERT gene expression (Figure 29). This TERT-positive sample, 305195 is a mast cell tumour case.

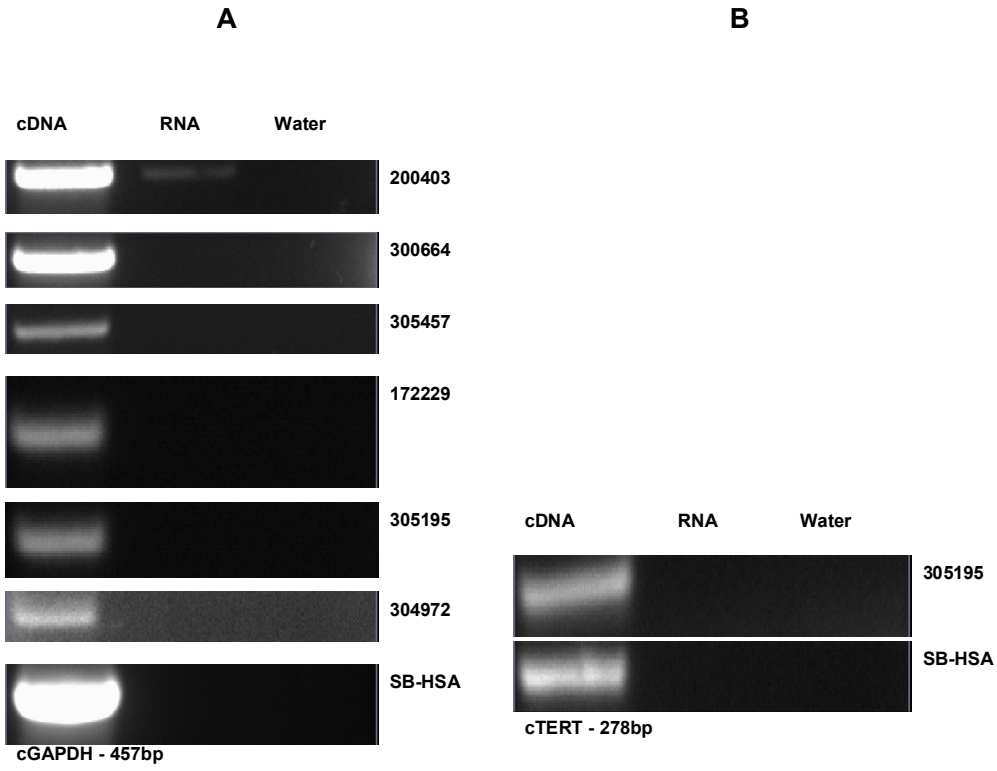


Figure 29 – Positive Serum Samples of cGAPDH or cTERT in Conventional PCR

A, cGAPDH expression in serum samples

B, c TERT expression in serum samples

CDNA, RNA and Water lane are the same as defined above (Figure 21).

4.4.6 Result of Samples subjected to Real-time PCR Test

In a total of 26 samples subjected to real-time PCR analysis, eukaryotic 18S ribosomal RNA expression was found in all samples with Cp values ranging from 20.69 to 34.99. Canine GAPDH gene expression was found in 23 out of the 26 samples with Cp values ranging from 31.20 to 37.26. However, canine TERT gene expression was not detected in any of the 26 samples (Table 19). 200403, 300664 and 172229 which were subjected in the previous conventional test, were also tested in the real-time PCR system and they showed the same cGAPDH-positive and cTERT-negative result.

Case Number	18S Av Cp	cGAPDH Av Cp	c TERT Av Cp
SB/HSA cells	10.2	19.29	29.87
304610	32.11	-	-
304327	27.55	35.83	-
304364	24.3	34.09	-
303505	20.69	32.58	-
304779	21.64	-	-
304022	23.43	34.62	-
303878	22.26	34.23	-
204205	33.01	-	-
304163	20.74	36	-
304819	24.12	33.91	-
301309	31.17	37.26	-
304395	25.02	34.43	-
200403	21.69	31.97	-
300664	21.7	31.96	-
172229	25.29	33.38	-
305214	28.03	35.03	-
303060	20.94	31.2	-
304299	24.56	33.64	-
204230	25.74	34.79	-
304882	24.95	35.03	-
204578	22.31	32.3	-
201763	29.92	36.73	-
305759	24.63	33.98	-
203399	27.2	34.48	-
303166	34.99	38.66	-
302093	24.15	33.24	-

Table 19 - Results of Serum RNA Subjected to Duplex Real-time PCR

Av Cp stands for average Cp value and it was calculated from the triplicates. Only serum samples with three clear Cp values were included in the table. Blank cells mean that no Cp value was detected.

4.4.7 Summary of Serum RNA Result

In total 36 canine serum samples from the oncology department were investigated. Apart from case 304395 which was diagnosed as penile bleeding trauma, all other cases were diagnosed with a variety of canine cancers including mast cell tumour, lymphoma, squamous cell carcinoma, adenocarcinoma, leukaemia, histiocytoma,

splenic sarcoma, lipoma, transitional cell carcinoma and round cell tumour. 10 serum RNA samples were subjected to conventional RT-PCR test. 23 serum RNA samples were subjected to real-time PCR. 3 serum RNA samples were able to be subjected to both tests because in the beginning of RNA extraction, 50µl NF-water instead of 30µl was used for elution of the extracted RNA from these serum samples. 18S rRNA were found in all samples that were tested. The cGAPDH mRNA was present in 26 out of 36 samples and cTERT was present in only 1 out of 36 samples (Table 20).

Case Number	18S rRNA	cGAPDH	cTERT	Clinical Case
305195	NA	+	+	Mast Cell Tumour
200403	+	+	-	Multi-site Mast Cell Tumour
300664	+	+	-	Mast Cell Tumour
172229	+	+	-	Squamous Cell Carcinoma
305457	NA	+	-	Mast Cell Tumour
304972	NA	+	-	2-sites Mast Cell Tumour
304404	NA	-	-	Squamous Cell Carcinoma
201603	NA	-	-	Multi-site Mast Cell Tumour
305178	NA	-	-	Histiocytoma
309300	NA	-	-	Lymphoma
304439	NA	-	-	Lymphoma
304925	NA	-	-	Squamous Cell Carcinoma
204763	NA	-	-	Mast Cell Tumour
304327	+	+	-	Round Cell Tumour
304364	+	+	-	Round Cell Tumour
303505	+	+	-	Multicentric Lymphoma
304022	+	+	-	Mast Cell Tumour
303878	+	+	-	Multicentric Lymphoma
304163	+	+	-	Adenocarcinoma
304819	+	+	-	Mast Cell Tumour
301309	+	+	-	Mast Cell Tumour
304395	+	+	-	Penile Trauma-caused Bleeding
305214	+	+	-	Lipoma
303060	+	+	-	Transitional Cell Carcinoma
304299	+	+	-	Aculte Lymphoblastic Leukemaia
204230	+	+	-	Adenocarcinoma
304882	+	+	-	Cranial Tumour
204578	+	+	-	Mast Cell Tumour
201763	+	+	-	Cervical Tumour
305759	+	+	-	Mast Cell Tumour
203399	+	+	-	Splenic Sarcoma
303166	+	+	-	Lymphoma
302093	+	+	-	Lymphoma
304610	+	-	-	Parathyroid Tumour
304779	+	-	-	Squamous Cell Carcinoma
204205	+	-	-	Mast Cell Tumour

Table 20 – Summary of all Serum Sample Results

Samples that were subjected to conventional RT-PCR do not have 18S rRNA data, an this is shown as NA (not applicable).

4.5 Discussion

In this study, I have shown that 18S rRNA can be detected in all canine serum

samples, which is consistent with the findings in humans (Dasi et al., 2001). I have also shown that cGAPDH mRNA can be detected and amplified in most of our serum samples, which is also consistent with publications in human studies (Miura et al., 2003, Wong et al., 2004, Li et al., 2006, Rykova et al., 2006). From the Cp value gap between TERT mRNA and 18s rRNA of SB-HSA cells (19.67), the expected cTERT Cp value of our samples is between 40.36 and 54.66, which is beyond the detection limit of our real-time PCR machine. Therefore, even if circulating cTERT mRNA does exist in the serum of canine cancer patients, the real-time PCR would not be able to detect it mainly because of the poor quality and quantity of RNA. In addition, even if circulating cTERT mRNA could be detected in canine cancer patients, it would still be a challenge to translate the whole process into clinical practice at this stage. An effective cancer biomarker should not only be present at significant levels for identification, but the process of detection should also be practical in real clinical circumstances. Not only do all steps involved in processing blood samples need to be done in a well-equipped lab, but also every step requires the attention of an experienced technician in order to protect the samples from RNA degradation and to avoid contamination. In addition, the process of biomarker detection should be cost-effective, and this currently cannot be achieved in reality. Thus the complexity involved from the beginning of obtaining blood samples to the end of analysing the level of TERT transcripts in serum makes circulating mRNA very unlikely to become an effective cancer biomarker in the near future.

As it had previously been shown that in order to obtain stable serum RNA concentrations, clotted blood should be stored at 4˚C and processed within six hours (Tsui et al., 2002), I aimed to process all blood samples immediately after collection. However, on several occasions blood samples was left at room temperature for up to 1 hour before being processed but no significant difference in subsequent RNA yield and quality was observed when compared with the blood samples that were processed immediately after collection.

There have been many protocols reported for isolating serum or plasma from blood samples in order to gain a pure cell-free solution. Some early studies only used one

centrifugation step to isolate serum or plasma from blood samples (Silva et al., 2001, Kopreski et al., 2001). However, it has been reported that a single centrifugation step cannot efficiently spin down all the platelets (El-Hefnawy et al., 2004), which are also known to contain RNA (Martincic et al., 1999, Bugert et al., 2003) and would therefore contaminate the cell-free serum mRNA of the samples. Thus, I adopted the three centrifuge step protocol (Miura et al., 2003) to ensure that I obtained as pure a cell-free serum as possible. This protocol uses 1,000 x g, 1,500 x g and 3,000 x g at each 10min centrifugation step. Each step was followed by careful harvesting of serum and transfer of serum to new RNase-free tubes. Interestingly, it was reported that filtering plasma samples from cancer patients through a 22μm filter before RNA extraction resulted in a detectable human GAPDH mRNA signal, and that a small signal of GAPDH was still observed even after ultracentrifugation (99, 960 x g) of the plasma. This data thus questions the particle-associated nature of circulating RNA, as the ultrahigh speed force would be expected to pellet virtually all particulate matter (Ng et al., 2002). It has also been reported that after filtration of plasma samples through a 0.22μm filter, a clear difference in hGAPDH expression was observed between healthy controls and patients with minor, moderate or severe trauma injuries and patients who died (Rainer et al., 2004), where the median filtered plasma GAPDH concentration was increased more than 7 fold in all injury groups. This evidence suggests that circulating RNA may exist in another form distinct from the particle-associated form.

To ensure our findings truly reflect the nature of circulating RNA, apart from careful handling of RNA in general, we used three experimental strategies. Firstly, the 3-step spin protocol ensured a cell-free serum environment as any cellular RNA contamination would change the gene profile of the circulating RNA. Secondly, any extracted Serum RNA was subjected to a Dnase treatment to avoid any genomic DNA contamination carried over to the downstream PCR analysis. Lastly, I used intron flanking primers to identify amplification of contaminating genomic DNA. With all these three controls, the PCR result on circulating RNA would be more realible.

It has been observed that repeated freeze–thaw cycles promote degradation of RNA in serum (Kopreski et al., 1999) but that a single freeze–thaw cycle does not have a significant effect on RNA concentration in serum or plasma (Tsui et al., 2002, Cerkovnik et al., 2007). Therefore, all of our serum samples were frozen at -80˚C immediately after centrifugation and processed for RNA isolation immediately after thawing.

As currently there is no standard method of isolating circulating RNA from human serum or plasma, and there have been more than ten methods published using existing RNA isolation technique with modifications, I decided to try 3 different total RNA isolation methods. The Sigma TRI BD reagent was chosen because it was reported to be the most efficient way to isolate circulating RNA from large volumes (in ml quantity) of serum or plasma (El-Hefnawy et al., 2004). In fact I was able to isolate detectable and amplifiable circulating RNA using all three methods. However, the Promega SV Total RNA Isolation system could only process up to 100µl serum samples. Although I successfully used the Roche High Pure Viral RNA Kit to process serum samples of up to 600µl, the kit was designed only to process 200µl serum at a time, and thus required the sample to be loaded three times at each step. I found that this procedure was not only time-consuming, but also increasing the risk of contamination. Although the Sigma TRI BD method can potentially process large volume serum samples, I found it very difficult to isolate the tiny RNA pellets from the large aqueous phase. As a result, total RNA isolated from almost all samples using all three methods above had very low quantity as well as poor quality. However, it was reported that circulating hTERT mRNA could be detected in as little as 50µl of serum from human cancer patients (Miura et al., 2003), but I could not detect cTERT mRNA in most of our canine serum samples of volumes ranging from 100µl to 3.6ml. This may be explained by either the different amounts or mechanisms of circulating RNA in humans and dogs.

After quantifying the total RNA purified from serum, the 260/280 absorbance ratio that indicates the quality of RNA was extremely low. This could have been the result of using suboptimal methods of circulating RNA isolation or due to the existing form

or forms of the RNA itself. Interestingly, there have been reports indicating that the circulating RNA detected in human plasma is highly fragmented and degraded (El-Hefnawy et al., 2004, Cerkovnik et al., 2007).

There also have been studies using GAPDH mRNA as control for total RNA in quantitative real-time PCR analysis (Wong et al., 2004), but an interesting result showed no correlation between GAPDH mRNA and 18S rRNA copy number and total RNA concentration in both healthy donor and breast cancer patients. This indicates that the process involved in the generation and circulation of circulating mRNA may not be universal and maybe different for specific RNA forms (Rykova et al., 2006).

Finally, in this study, all the dogs sampled were suspected cases of neoplasia. Among all the samples collected, 35 are canine neoplasia cases with one non-neoplasia case. Among the 35 neoplasia cases, 4 cases are benign tumour and 31 cases are malignancy.

Although the potential use of circulating TERT mRNA for canine cancer diagnostics is highly attractive, more questions regarding the mechanism involved in generating circulating RNA still need to be answered, and an effective standard protocol for isolating circulating RNA must be developed in order to detect the tiny amount of TERT transcript.

4.6 Conclusion

Although our study did not detect TERT transcript in the majority of serum samples from canine cancer patients, I did confirm that canine mRNA transcript are found in the circulation of dogs as in humans. However, whether circulating TERT does or does not exist in the serum of canine cancer patients, I found that it is currently not practical to utilize circulating mRNA as a cancer biomarker in dogs. As the methods and techniques of biomarker research and clinical application are largely transferable from humans to animals, and as more advanced methods are developed to detect circulating RNA in humans in the future, then we can expect to obtain a more

comprehensive understanding of the nature of these molecules and their validity as cancer biomarkers in humans and dogs.

CHAPTER 5

THE INVESTIGATION OF TELOMERASE INDUCTION IN CANINE SOMATIC CELLS: A PILOT STUDY

5.1 Chapter Abstract

Telomerase is not only crucial for tumour immortalization, but emerging evidence has also linked telomerase with ageing. Ectopic expression of TERT in a variety of telomerase-negative normal somatic cells has been shown to prolong cellular lifespan without any malignant transformation of the cells. Telomerase has been found to play an important role in stabilizing stem cell functionality which is crucial for maintaining tissue homeostasis and potentially organismal ageing. By using telomerase-null mice in many *in vivo* studies, the late generations of these mice with critically-short telomeres have shown many signs of premature ageing at the organ, tissue and cellular level. In the meantime, telomere length has also been investigated as a potential biomarker for ageing. Very recently, the screening of compounds and natural product extracts has identified several novel telomerase activators that can induce telomerase in normal cells and may thus provide an alternative strategy to study the role of telomerase in ageing.

In this study, three novel compounds provided by Sierra Company were tested for their ability to induce telomerase in telomerase-negative canine embryonic fibroblasts. A duplex real-time RT-PCR system was established to analyse cTERT expression level relative to the expression of 18S rRNA in cells.

A strong increase of cTERT mRNA level was found after 24 hours of compound exposure by compounds C00 and C01 but not C03. In addition, varying the dose of these compounds had no effect on the elevated cTERT levels.

Taken together I have shown that telomerase can be activated endogenously by novel compounds in normal canine somatic cells through elevation of TERT mRNA levels. As far as I know this is the first study of telomerase induction being investigated in the canine subject, and this study has also demonstrated the capability of novel telomerase activators in canine somatic cells, thus providing an alternative way to study the role of telomerase in ageing.

5.2 Introduction

In addition to reports indicating that telomerase can be activated by the ectopic expression of TERT, recent studies have shown that compounds and natural product extracts can activate endogenous telomerase.

Resveratrol (trans-3,5,4′-trihydroxystilbene), a natural polyphenol phytoalexin that possesses diverse biochemical and physiological actions, was found to significantly increase telomerase activity in endothelial progenitor cells through the PI3K pathway and to prevent the senescence of EPC (Xia et al., 2008)

Cycloastragenol (TAT2), a saponin derived from the herb *Astragalus* was found to slow down telomere shortening of $CD8^+$ T lymphocytes from HIV-infected patients through telomerase activation, and this effect was induced via activation of the MAPK pathway which subsequently increased hTERT mRNA and/or phosphorylated hTERT protein (Fauce et al., 2008). A similar effect was also reported using ectopic expression of TERT (Rufer et al., 2001).

Furthermore, TA-65 proprietary capsules, made mainly from extracts of the dried root of *Astragalus,* were found to moderately activate telomerase in human keratinocytes, fibroblasts, and immune cells *in vitro.* In addition, when taken as part of a dietary supplement program, patients taking TA-65 were found to have proportionally fewer immune cells with critically-short telomeres 3 months later compared to the start of the study and this effect continued until the end of study (12 months after taking TA-65) (Harley et al., 2010). In a mouse study, TA-65 was found to activate telomerase and elongate critically-short telomeres in mouse embryonic fibroblasts *in vitro*, to increase TERT levels in some tissues and to improve the healthy lifespan of mice when administered orally (de Jesus et al., 2011).

So far most studies regarding telomerase induction in normal cells were based on mouse or human studies, using either gene transfer of TERT or by administering a telomerase activator. In this small pilot study, I aimed to answer one important question: can endogenous telomerase be induced in canine normal somatic cells?

Thus for the first time, I tested three novel compounds for their ability to activate telomerase in telomerase-negative normal canine somatic cells. These compounds were a gift from the Sierra Science Company which identified novel telomerase activators by screening hundreds of thousands of compounds and natural product extracts.

5.3 Materials and Methods

5.3.1 Telomerase Induction in CEF Cells

Canine embryonic fibroblasts were seeded at a density of 2.5 x 10^4 in a 96-well plate containing 50µl medium per well. 24h after cell-seeding, several doses (20µM, 33µM and 50µM) of compounds C00, C01 and C03 were added to triplicate wells. As negative controls, mock treatment with DMSO and untreated cells were also included. Cells were allowed to grow for another 24h and then total RNA was extracted.

5.3.2 Duplex Real-time PCR to Detect Induced cTERT m RNA

Duplex real-time PCR was conducted to analyse levels of 18S rRNA and cTERT mRNA gene expression in these cells. Relative gene expression was calculated using 18S rRNA to calibrate samples. Results were obtained using the ΔΔCp method. In detail, ΔCp was firstly obtained using the TERT Cp value minus the 18S value. ΔΔCp was then obtained using the compound ΔCp value minus the DMSO-treatment Cp value. Finally, the $2^{\wedge(-\Delta\Delta Cp)}$ was calculated. Primer sets and running program were the same as that in the previous chapter (Table 12).

5.4 Results

5.4.1 CTERT Expression of Compound-treated CEF Cells

Canine embryonic fibroblasts were treated with three compounds: C00, C01 and C03. After 24 hours exposure to each compound, TERT mRNA expression level was seen to be significantly increased for both C00- and C01-treated cells, compared to

DMSO-treated cells, but not for compound C03-treated cells. The increased level of TERT mRNA in cells treated with compounds C00 and C01 did not differ significantly for the 20µM, 33µM or 50µM doses (Figure 30).

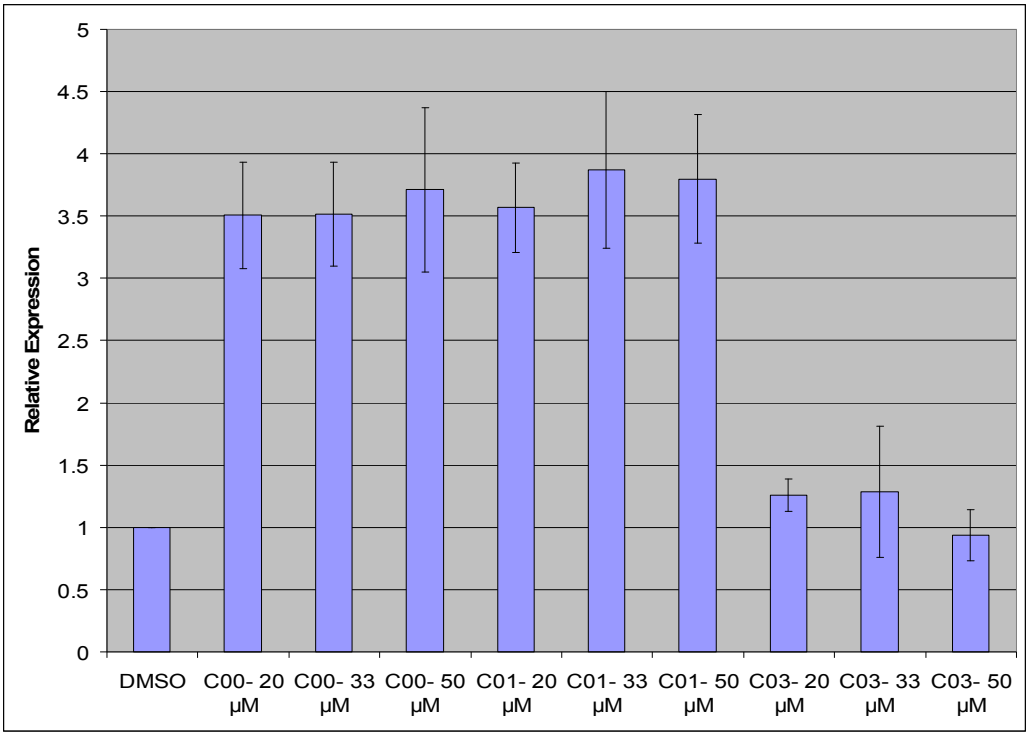


Figure 30 – Relative Expression of cTERT between Compound-treated and DMSO-treated CEF cells

After Real-time PCR, relative cTERT expression level was calculated against the expression of 18S r RNA.

5.5 Discussion

In this pilot study, three novel compounds were tested for their ability to induce cTERT mRNA in telomerase-negative primary canine embryonic fibroblasts. Although several studies have reported the capability of telomerase activators in normal somatic cells, none has clearly demonstrated a corresponding increase in TERT mRNA levels and none have used the sensitive quantitative real-time PCR method to investigate changes in TERT mRNA levels (Fauce et al., 2008, Xia et al., 2008, Harley et al., 2011, de Jesus et al., 2011). Here we report a dramatic increase in

canine TERT mRNA levels caused by a 24-hour exposure to compounds C00 or C01, but not C03, measured using a real-time duplex RT-PCR system.

There are always concerns regarding possible associated toxicity when using compounds as telomerase activators, as significantly elevated apoptosis has been observed in some reports (Xia et al., 2008). However, although doses up to 50 μM of the three compounds were used, no apparent toxicity effects were seen in these canine embryonic fibroblast cells.

I used 18S rRNA as a control gene, as 18S rRNA levels are relatively more consistent and its expression level is less likely to fluctuate under treatments that may affect the expression of mRNAs of some commonly used housekeeping genes. Part of the reason for this is that rRNA constitutes approximately 80% of a total RNA sample from eukaryotic cells, and therefore the concentration of a total RNA sample determined by spectrophotometry is already normalized to the amount of rRNA present. Second, rRNA is transcribed by RNA polymerase I (Russell and Zomerdijk, 2005) which is different from the polymerase used for mRNA transcription, and thus its expression may be differentially regulated. Interestingly, several reports have shown that the expression level of rRNA remained stable while levels of β-actin and/or GAPDH gene varied in individual rat livers (de Leeuw et al., 1989), in some breast cell lines (Spanakis, 1993), human skin fibroblasts (Mansur et al., 1993) and in some malignant mouse cell lines (Bhatia et al., 1994).

As most studies have used the genetic transfer of TERT to activate telomerase, several concerns have been noted. First, gene therapy applications may take longer to be translated into clinics due to significant safety concerns, especially when using viral vectors and the subsequent inability to control the level and extent of telomerase induction in the whole body. On the contrary, compound-based telomerase activator application can be conducted in a dose- and duration-controlled manner, and some evidence has shown that drug-induced telomerase activation can be short term and reversible without any effects on cell viability (Fauce et al., 2008). Second, the introduction of telomerase bears the risk of inducing cancer, as has been found in

some *in vivo* studies using ectopic TERT expression (Gonzalez-Suarez et al., 2005, Donate and Blasco, 2011). However, so far studies conducted on telomerase activators have shown that they did not significantly increase the intrinsic telomerase activity in tumour cells nor promote any loss of control or malignant transformation (Fauce et al., 2008).

5.6 Conclusion

Despite being a small pilot study, I have clearly shown for the first time that telomerase can be activated in telomerase-negative canine somatic cells through upregulating TERT mRNA levels and that telomerase can be induced by two novel compounds. This study has brought more knowledge to the current study of telomerase induction. In the future, it will be important to further investigate and characterize the telomerase induction effect. It would be interesting to explore whether telomerase induction is transient and if there is an immediate or long-term effect on telomere length and cellular lifespan. Also it would be intriguing to explore whether the same effect can be found in other canine cell types including tumour cells and eventually also in animal experiments. Taken together, as a novel approach, this non-genetic compound-based telomerase activation strategy may provide an alternative way to study telomerase and its role in ageing.

CHAPTER 6

FUTURE PERSPECTIVES

As cancer still tops the list of deadly diseases in both humans and dogs, developing a method for cancer early detection as well as finding a way to cure it have been the absolute priorities in scientific research. Telomerase, which represents a key enzyme in cancer immortalization, holds great promise as an attractive cancer biomarker and anticancer target. In addition, beyond cancer, increasing evidence has highlighted its role in ageing. Since its discovery, telomerase has been intensively studied for more than two decades. Although its discovery has changed our way of seeing cancer, we still have not gained a comprehensive understanding of this enzyme or a clinical solution on how it may be targeted in order to cure cancer. Currently, most of cancer-based research has relied on rodent tumour models. However the question of whether data obtained from these models can truly reflect human cancer remains to be answered. Canine cancer, one of the most deadly diseases in dogs with a high incidence estimated at 1437 per 100,000 dogs per year for skin and soft tissue cancer in UK (Dobson et al., 2002), represents a potential new model to study telomerase and telomeres. Unlike mice and other rodents, canine cancer contains a wide range of naturally-occurring tumours and canine telomere length is also comparable with humans.

As an efficient delivery system is essential for any anticancer therapeutic application to treat tumours systemically, I explored two novel systemic delivery systems in the canine context in order to gain a better understanding of their efficacy *in vitro* and *in vivo*. Both cellular vehicles and synthetic polymers have their own advantages. Cellular delivery vehicles as a novel strategy to target tumours systemically with a transgene have been proved feasible. As I have shown in this study, the canine malignant endothelial cell type SB-SHA cells can be manipulated easily to express a foreign gene *in vitro*, however their *in vivo* tumour-homing ability is still not clear. Currently there is still debate regarding the concept of endothelial lineage cells, especially endothelial progenitor cells, and their tumour homing ability, and thus further studies are needed to understand their nature *in vivo*. If possible, other canine endothelial cell types should also be investigated in the future *in vitro* and *in vivo* for their potential tumour-homing ability. If proven, these cellular delivery vehicles will be ideal to treat primary tumour as well as distant metastases.

The PPIG3 dendrimer is an emerging polymer vector that has recently been developed as both a systemic delivery vehicle as well as for its intrinsic antitumour activity. In this study we have tested its gene transfer ability and observed its low transfection efficiency with canine cells compared with commonly-used Lipofectamine™2000. The low transfection rate with canine cells suggests further improvement of this vector as this could hinder its efficacy when used systemically. As these polypropylenimine dendrimers are synthetic polymers, further development should also be focused on structurally modifying these dendrimers to improve their gene transfer ability without affecting their unique property of forming nanoparticles with DNA that are adapted to the EPR effect. In addition more research is required to further prove their specific tumour accumulation ability *in vivo* and the EPR effect which it is based on. In the future, it would be also very interesting to further explore their intrinsic antitumour activity as well as their potential influence on cellular gene expression to assist the development of new generations of these dendrimers as systemic delivery vehicles.

After having explored two novel delivery systems, I then constructed a plasmid-based siRNA vector targeting canine telomerase RNA component and tested it on two canine cancer cell lines. Since the discovery of RNAi, it has been utilized to inhibit specific gene expression by synthetic siRNA molecules *in vitro* and *in vivo* and this has become a common and standard method in the laboratory. The recent first in-human phase I clinical trial of systemically-administered siRNA has further proved its ability for specific gene inhibition at both mRNA level and subsequent protein level; thus siRNA technology is still a popular and attractive strategy for anticancer applications. Although using siRNA to target telomerase is not a new idea, scientific publications on canine telomerase itself are very limited, the inhibition of canine telomerase by siRNA which has also been rarely documented. In this study, I designed and constructed a plasmid-based siRNA expression vector, since not only does this vector plasmid-based vector have many advantages over viral or naked siRNA, it can also be combined with the two novel delivery vehicles we explored earlier. In the siRNA experiment, I did not observe any gene-silencing of cTR,

however, it did not necessarily suggest that cTR can not be targeted by siRNA. Although this plasmid-based siRNA vector has been designed to induce gene-silencing in mammalian cells, only gene-silencing in human cells has been demonstrated so far. Future studies should investigate its ability to induce gene-silencing in canine cells to validate its efficacy. I also have noticed the difference of the minimal length of target mRNA required in different siRNA expression systems and this could also play a part in the failed cTR gene-silencing. In the future, more target regions on cTR gene using the siRNA vector should be established and the potency of these regions for RNAi induction should be compared

Telomerase not only represents a potential anticancer target, but is also considered a near-universal tumour marker. As currently there are urgent needs for a specific, accurate and informative biomarker in cancer clinics not only for diagnostics, but also ideally for tumour staging as well as post-treatment monitoring, cancer-specific circulating mRNA has been explored as a promising candidate. If proven it would be an ideal biomarker for minimally invasive cancer detection and progression with repetitive monitoring from blood. However although circulating TERT mRNA has been found in human cancer patients and several reports have highlighted its potential diagnostic value and prognostic value, I did not identify TERT mRNA in the circulation of the majority of canine cancer patients in this study. However this finding does not necessarily preclude the existence of TERT transcripts in the circulation of cancer-bearing dogs but alternatively suggests the possibility that failed detection using the current methods is due to its extremely low expression level as to the complexity of the isolation process of serum RNA which prevented its detection in our system. Therefore, circulating canine TERT mRNA cannot be utilized as a cancer biomarker currently. As recent studies have also concluded that the amount of mRNA found in serum or plasma of cancer patients is highly variable among different published papers (Schwarzenbach et al., 2011), future studies should not only focus on the extraction method of these RNA transcripts, their nature should also be investigated in order to evaluate their suitability as biomarkers. As experimental techniques and methods for RNA extraction and molecular detection are advancing year by year, it might be even possible to detect them straight from

serum or blood without an extraction step in the future. Lastly similar studies should be performed again to re-evaluate their existence in canine cancer patients.

Human life expectancy has nearly doubled in the last century because of medical advances; however there is still a lack of a complete understanding of the biological processes and mechanisms underlying ageing and ageing-related disorders. Much evidence has shown that beyond cancer, telomerase and telomeres function not only in cellular ageing but also possibly in organismal ageing. Although a pilot study, for the first time our finding of the endogenous expression of TERT being induced by novel compounds clearly demonstrated that telomerase can be activated in canine normal somatic cells. Future work should follow up this discovery to determine the effect of telomerase induction on the lifespan of CEF cells as well as on other canine somatic cells. It is also important to investigate the mechanism underlying this drug-induced telomerase induction. After that, *in vivo* experiments could also be conducted to see if these compounds have influence on health and lifespan of animals. Fundamentally, understanding the role of telomerase and telomeres in the process of overall ageing would definitely help us to contribute to the fight against cancer.

Taken together, as cancer continues to be a common and deadly disease in dogs and humans, there are growing requirements for novel anticancer drug targets, as well as molecular biomarkers for diagnosis in clinics. Thus understanding canine telomerase will not only benefit veterinary oncology, but will also provide a comparative model system to help us understand the fundamental role it has in human cancer and ageing.

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